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REVERSAL OF EVOLUTION IN THE GENUS PENSTEMON*

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INTRODUCTION

The hypothesis is offered that evolution is, in a sense, reversible and that widely divergent germplasms may be returned to a common gene pool. Further, it is suggested that from such a gene pool, the worker may isolate divergent lines that he, within his working lifetime, might use to produce true breeding forms that could, by present taxonomical standards, be described as new species.

At the North Platte Experiment Station of the University of Nebraska, breeding experiments in the genus Penstemon show that the establishment of such a gene pool is possible. Through a series of intersectional hybrids that have been intercrossed in a number of combinations, a panmictic population that combines germplasms of six sections of the genus has been produced. (See figure 1). Additional intersectional hybrids have been made and the prospect of combining germplasms of many species belonging to a number of sections of the genus, is near realization.

The intersectional hybrids produced are highly fertile and, more important are proving interfertile. Factors for genetic isolation of species within sections and between species belonging to different sections of the genus are, in some manner, being inactivated in the hybrid material. Germplasms incompatible in direct crosses are being combined through the medium of a third species, compatible to both. At the present stage of the breeding experiment, a number of hybrids that combine germplasms of three or more sections of the genus have been produced and have fruited.

Much of the parental material used in this hybrid series is so different morphologically that the question of its specific identity cannot be raised. To do so would impute doubt of the validity of our present taxonomic system. Many of the species that have been combined in the breeding program are genetically isolated to the degree that repeated attempts to cross them directly have failed to produce a single viable zygote; yet, germplasms of these incompatible species have been combined in interfertile hybrids

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through a third intermediary species belonging to a different section of the genus.

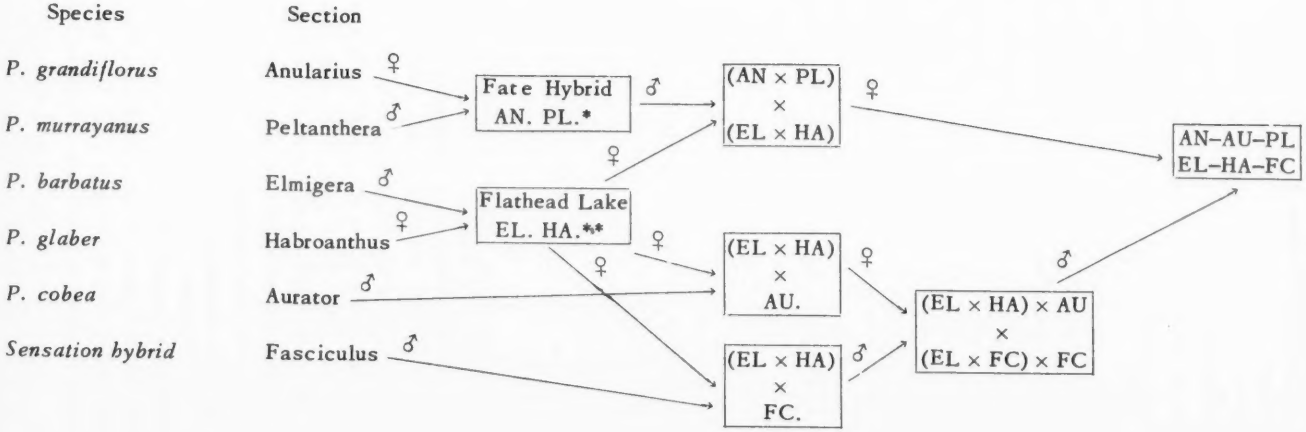
While the present exploration has not determined the extent to which genetic isolations may be bypassed through the media of intermediary hybrids, it seems highly probable that germplasms of most of the species of the large seeded sections: Anularius, Aurator, Elmigera, Fasciculus, Habroanthus and Peltanthera may be returned to a common gene pool. Further, there is the probability that a similar gene pool may be established by hybridization of the small seeded sections of the genus, and even a possibility that barriers between the large and small seeded sections may be crossed. Regardless of future developments, the present interfertile, intersectional hybrids offer an unusual opportunity for evolutionary investigation. With the broad genetic base inherent in the combination of germplasms of a number of sections of the genus, such a gene pool should be a fertile source of divergent lines that might add much to our knowledge of evolution.

LITERATURE REVIEW

A search of the literature has failed to discover any reference mentioning the concept of returning a number of divergent germplasms to a common gene pool or of the use of such a gene pool as material for the study of the phenomenon of evolution. Clausen, 1952, states, "The plant breeder and evolutionist alike have greater opportunity to produce entirely new forms fitted for very contrasting econiches if they are able to draw upon the genetic resources within a major part of a genus rather than being limited to the variability of a single species." In this statement, he implies, but does not state, the potential value of the complex gene pool for evolutionary investigation. Anderson, 1949, draws attention to the ecological significance of combining divergent germplasms and is emphatic regarding the vast number of ecotypes to be expected in the segregating generations of species hybrids; while DeGraaff, 1951, senses the value of the species cross as a source of new plant forms and recognizes the potential of the species hybrid as a vehicle for joining incompatible germplasms.

The literature of the interspecific hybrid is voluminous and its use in evolutionary study is becoming well documented. These studies are, however, mainly concerned with analysis of the segregating generations of single hybrids or at best a few hybrids. The literature search failed to reveal use of the concept of the complex gene pool as a tool for evolutionary research.

The swarms of interspecific hybrids that appear in nature have been used for evolutionary research and excellent work has resulted, for example Riley, 1938, in an analysis of hybrid Iris, and Hieser, 1947, in his Helianthus studies. In Penstemon, Faith Mackaness, 1957, reports (a) swarms of hybrids between species of section Dasanthera and (b) evidence of introgressive hybridization between species of Saccanthera in the Columbia River Gorge, near Troutdale, Oregon. She also reports indication of polyploidy appearing in hybrid populations. Straw, 1955, suggests that hybrids



* Fifth generation hybrid from controlled cross.
** Natural hybrid

Abbreviations of sectional names used: AN—Anularius; AU—Aurator; EL—Elmigera; FC—Fasciculus; HA—Habroanthus; PL—Peltanthera.

FIGURE. 1. Breeding method used to return germ plasms of six sections of Penstemon to a common gene pool.

of sympatric species may, under certain circumstances, become established as separate entities. He also states, 1956, that "Closely related species of Penstemon, if four species in section Peltanthera are at all typical, seem to have the ability to cross with ease."

While Straw's observation concerning hybridization between species of section Peltanthera have been verified, his assumption that closely related species may cross with ease, has not, in the author's experience, held for member species of other sections. Sympatric species native to the Northern Rocky Mountains and adjacent Great Plains appear so well isolated, genetically, that gene exchange is rare. Repeated attempts by the author to cross these sympatric species, regardless of relationship, have failed to result in a single hybrid.

While no one questions the value of the hybrid swarm and the interspecific hybrid as tools for the study of evolution, such material does not lend itself well to the idea of controlled evolution. In the hybrid swarm, as pointed out by Anderson, 1949, only a few of the possible genotypes establish themselves. Ecological niches for all possible genotypes simply do not exist. In the interspecific hybrid, the genetic base is seldom broad enough to provide an adequate source of variation for more than a limited investigation. It would appear that the much broader genetic base provided by a complex hybrid population derived from a number of intersectional crosses has far greater promise to the student of evolution.

Further, the author wishes to point out that the cultivated rose, chrysanthemum and a number of other ornamental plants have developed from such gene pools as that proposed in his hypothesis. Admittedly, temporal aspects differ in that species germplasms were added to the latter gene pools in succession, over long periods of time. Nevertheless, principles involved are similar.

MATERIALS AND METHODS

A germplasm reservior consisting of about sixty Penstemon species, two natural hybrids, and a series of hybrids resulting from controlled crosses has been established at the North Platte Experiment Station. These hybrid materials, with only one or two exceptions, are intersectional hybrids. See table 1.

Both hybrids and species are being tested for cross compatibility in many combinations. Early hybrids have been crossed to produce complex hybrids that combine germplasms of three or more sections of the genus. Most of the combinations produced so far are interfertile and make it possible to combine germplasms of a number of sections in a single panmictic population.

In order to produce a population with the widest possible genetic base, additional intersectional crosses are being made each season. This program will continue until adequate germplasms have been united to provide the means of establishing any sort of gene pool the plant breeder or researcher may desire.

TABLE 1

LIST OF INTER AND INTRASECTIONAL SPECIES HYBRIDS OF PENSTEMON IN THE GERMPLASM RESERVIOR AT THE NORTH PLATTE EXPERIMENT STATION

GROUP ONE: Advanced Generation Hybrids

Flathead Lake: A natural hybrid with the putative parentage *P. barbatus* × *P. glaber.* Discovered near Flathead Lake, Montana, probably F_5 or later generation material. Sections Elmigera and Habroanthus.

Seeba Hybrid: A natural hybrid that occurred in a garden at Cook, Nebraska with *P. grandiflorus* × an unidentified species of section Peltanthera as parents. F_5 or later. Sections Anularius and Peltanthera.

Fate Hybrid: A controlled hybrid *P. grandiflorus* × *P. murrayanus,* sections Anularius × Peltanthera. Produced by Fred Fate, Columbia, Missouri. F_5 or later.

P. canescens × *P. digitalis:* Chance hybrid occurred in garden of Fred Fate. Possibly F_2, section Graciles.

P. cobea × *P. triflorus*: Advanced generation, F_5 or later, of original cross made by Mrs. J. Norman Henry, Gladwyne, Pennsylvania, section Aurator.

Sensation Hybrid: Exact parentage unknown, but morphology mostly that of section Fasciculus.

GROUP TWO: F_1 hybrids produced at the North Platte Experiment Station.

F_1 Hybrid	Sections Represented	Fertility*
Flathead × *P. alpinus*	(El. × Ha.) × Ha.	High
Flathead × *P. glaber*	(El. × Ha.) × Ha.	High
Flathead × *P. speciosus*	(El. × Ha.) × Ha.	High
Flathead × *P. strictus*	(El. × Ha.) × Ha.	High
Flathead × *Seeba Hybrid*	(El. × Ha.) × (An. × Pl.)	High
Flathead × *Fate Hybrid*	(El. × Ha.) × (An. × Pl.)	High
Flathead × *P. cobea*	(El. × Ha.) × Au.	Partial
Flathead × *P. barbatus* var. *Mullberry*	(El. × Ha.) × El.	High
Flathead × Sensation Hybrid	(El. × Ha.) × Fc	Partial

*Crosses classed as high in fertility will produce 95–100% fruiting plants; those indicated as partially fertile produce less than 95% fruiting plants.

Note: Abbreviations used: An.—Anularius, Au.—Aurator, El.—Elmigera, Fc.—Fasciculus, Ha.—Habroanthus, Pl.—Peltanthera.

It is assumed that all species, including *P. cobea*, used in the breeding program have been diploid, N = 8. While Penstemon cobea has been reported as being an octaploid species, Darlington and Ammal, 1945, the strain used in the present breeding program has proved to be diploid, Weiler, 1958. This form of *P. cobea* is an aberrant one introduced to the American Penstemon Society in 1954 by Miss Minnie Raabe of Temple, Texas. The Raabe cobea has white flowers, paler foliage, more slender stems and blooms later than typical *P. cobea.* Weiler has counted the chromosomes of the pollen mother cells of the Raabe *P. cobea*, the clone of Flathead Lake with which it was crossed and the resulting hybrid, Flathead Lake × Raabe *P. cobea*, and all counts were diploid. Meiosis has been regular and fertility of both hybrid and parental species is high. There is no basis for the assumption of the

American Penstemon Society that the Raabe form of *P. cobea* may itself be hybrid.

DISCUSSION

The present program is based upon the fortuitous discovery that a Penstemon known by the vernacular name 'Flathead Lake,' and which by taxonomic characters belongs to section Elmigera, produces hybrids when fertilized with pollen of species belonging to various sections of the genus. All hybrids produced so far with Flathead Lake as the female parent are quite fertile as well as being interfertile.

Flathead Lake is presumed a natural hybrid, with *P. barbatus*, Nutt. and *P. glaber*, Hook as the putative parents. This Penstemon was discovered near Flathead Lake, Montana, by George Murray, and introduced to the American Penstemon Society by Anna C. Johnson 1956 of Butte, Montana. It is widely grown by members of the society and is beginning to appear in commerce.

At the North Platte Experiment Station, it has been successfully crossed with *P. secundiflorus*, Benth. of section Anularius; *P. cobea*, Nutt. and *P. eriantherus*, Pursh. of section Aurator; *P. barbatus*, Nutt. of section Elmigera; and *P. alpinus*, Torr., *P. glaber*, Pursh., *P. neomexicanus*, W&S, and *P. strictus*, Benth., all of section Habroanthus, and with the Anularius-Peltanthera hybrid *P. grandiflorus*, Nutt. × *P. murrayanus*, Hook.

In addition to the Flathead Lake hybrids, the Fate and Seeba hybrids of sections Anularius × Peltanthera have been crossed with a number of other species and seed has been produced. A third series of crosses within the small seeded sections of the genus has resulted in the complex hybrid (*P. canescens*, Britt. × *P. digitalis*, Nutt.) × *P. hirsutus*, Willd. This is being used as a female parent in an effort to combine germplasms of additional species of the small seeded sections of the genus. Efforts to cross the large and small seeded sections of the genus have been inconclusive. Seed has been produced and a few seedlings grown, but the morphology of these fail to indicate hybridity.

Compatibility Relationships

Interspecific incompatibility appears to be general between the sympatric species of the Great Plains and Northern Rocky Mountains, but less general in sections Elmigera and Peltanthera of the Southwest Deserts.

In Peltanthera, natural hybrids are not uncommon and a number of such hybrids are reported by Straw, 1956, and Everett, 1950. Likewise hybrid swarms occur where species of section Dasanthera overlap as is reported by Mackaness, 1957, and as was observed by the author in the Cascade Mountains of Oregon.

With the exceptions noted above, cross compatibility between sympatric species appears limited. In many species, even those belonging to the same section of the genus, genetic isolation appears so well developed as to

make gene exchange rare or impossible in natural or controlled matings. Sympatric species of the Northern Rockies and the Great Plains have failed to produce viable seed in repeated attempts by the author to cross them. It appears, however, that genetic isolation between these species is not always complete. The author has observed, in garden grown material, forms that appear to be the result of introgressive hybridization. From this evidence, he concludes that occasional fertile hybrids must occur. At present, it is known that a number of northern species are cross compatible with certain species native to the southwest. The *P. barbatus* Hybrid, Flathead Lake, has hybridized readily with a number of species as shown in Table 1. *P. grandiflorus* of the plains has crossed with *P. murrayanus* of the southwest to produce the relatively "true breeding" Fate Hybrid, which with the similar Seeba hybrid is now appearing in commerce. ("True breeding," as used here, refers to the fact that advanced generation hybrids are phenotypically very like the F_1 hybrid. In no sense does it imply genotypic uniformity.)

Factors for genetic isolation appear to be inactivated in hybrid germplasms. Species, incompatible in direct crosses, have been mated with a third species belonging to another section of the genus to produce hybrids that cross readily and are highly fertile. This high degree of fertility appears constant even when three or more sections of the genus are united in a complex hybrid. It must be recorded, however, that nonfruiting, and presumably sterile, individuals have been observed in most of the larger, hybrid populations. Such sterile individuals are rather uncommon and have not prevented the establishment of any complex hybrid population that has been attempted.

The Hypothesis of the Common Gene Pool

The concept of a gene pool in which germplasms of widely divergent taxonomic species are combined in a single panmictic population is a logical one. A number of combinations combining germplasms of three or more sections of the genus have been made. The composition of the gene pool or crossing block can be made static at any level desired by the investigator by the simple expedient of maintaining its components by asexual propagation. It can be modified at his discretion by the addition or subtraction of individual clones. It can be manipulated to produce any desired combination of germplasm without being modified by the genetic drift occurring in sexually maintained populations. Thus, it should provide an accurate tool for the study of evolution. The scientist can return again and again to the basic material of his investigation with the certainty that it is unchanged.

The idea of the static gene pool or the gene pool that can be manipulated with precision by the researcher may be a departure from the usual concept. It does, the author believes, offer the possibility of more precise control as well as a system of checks that cannot be duplicated in sexual populations. By the withdrawal or addition of elements from the breeding population the

contribution of any component can be measured. By precise manipulation, he can determine the genetic composition of divergent lines. In short, it offers the opportunity of "trial parties" limited in number only by the number of discrete elements in the breeding population.

The static gene pool can be maintained at any level of the breeding program. Species, F_1 hybrids, advanced generation hybrids and complex hybrids, in every possible combination may be used in its construction. Its contribution to the study of evolution will be that it provides a precise system of checks and counterchecks. Beyond this potential for controlled recombinations and the system of check and countercheck, it has no evolutionary significance.

Evolution is, in the final analysis, the result of sexual reproduction, of recombination, of mutation, of genetic drift and of the interaction between organism and environment. The static gene pool and the gene pool that can be precisely manipulated by the worker are only tools he may find useful in his research effort.

Evolutionary Significance

The production of a series of intersectional hybrids in Penstemon and the inactivation of the system of genetic and spatial isolation that enables species to exist as separate entities in nature, appears significant. The return of diverse germplasms to a common gene pool, or, if the reader prefers, to a panmictic population, in which the component individuals are interfertile and freely interbreeding may present an opportunity to study evolution under controlled conditions.

With the controlled evolution of divergent lines from such a gene pool, or a series of such gene pools, it is conceivable that natural processes requiring millenia might be duplicated within the scientist's working lifetime. The present series of hybrid Penstemon appears to offer such opportunity.

Admittedly, much more study and exploration of the possibilities are needed. Materials must be evaluated, combinations of germplasms tested, and actual divergent lines carried into advanced generations if the hypothesis of controlled evolution is to be proved or disproved. The inactivation of a system of genetic isolation, and the number of species in the genus with their wide morphological diversity, offers an almost unlimited number of possible germplasm combinations. The facility with which Penstemon germplasms are manipulated provides a unique opportunity for the researcher.

SUMMARY

The hypothesis that evolution is reversible and that widely divergent germplasms may be returned to a common gene pool is offered. Methods of combining germplasms of reproductively isolated species through an intermediary species as a vehicle are discussed. The gene pool that combines germplasms of a number of divergent species is considered as a source of new divergent lines which the worker may use to simulate natural evolutionary processes.

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usually occur among these progeny. Conjugation in Tetrahymena is regularly followed by a period of sexual immaturity which lasts for a variable number of cell divisions, but usually not less than 50. Tests of mating type are, therefore, not possible immediately following conjugation and all progeny of a single conjugating pair cannot be followed. Instead, the products of the first division (caryonides) are usually separated and are carried through the immature period by a series of single cell isolations. At maturity the terminal sub-clones are tested for mating types. Usually these sub-clones are pure for a mating type, but the mating types of caryonides from the same conjugating pair member are more or less independent. In contrast to this independence of sister caryonides, sister sub-caryonides (products of the second division following conjugation) are highly correlated. Thus, although all the offspring of a conjugating pair are alike in genotype, diversities in phenotype (mating type) regularly arise among them and the diversities are usually segregated at the first cell division following conjugation. Since the newly formed macronuclei separate at this same cell division, the phenotypic diversification within a genetically homogeneous population has been attributed to nuclear alteration. Since the original zygotic nuclei yield several types of progeny, they must initially be pluripotent. Hence, the diversification may be viewed as a restriction of potentialities through nuclear alteration.

Studies of the mating types arising at conjugation in various crosses show that the initial assortment of potentialities is controlled by nuclear genes (Nanney, Caughey and Tefankjian, 1955). One series of strains, designated as Family A, regularly produces mating types I, II, III, V and VI; another series (Family B) produces types II, III, IV, V, VI and VII. The heterozygote produces all seven mating types. The differences in potentiality appear to be controlled by genes at a single locus, the *mt* locus.

Although most of the caryonides examined at maturity are pure for a mating type, occasionally some (selfers) are found which are capable of conjugating in unmixed cultures and of producing in subsequent transfer cultures pure sub-cultures of diverse mating types. These caryonides have, therefore, failed to complete their differentiation, though some restriction of potentialities is indicated by the usual retention of only two potentialities in any particular clone. Since under certain conditions (maximal growth rate throughout the immature period) the frequency of such clones may reach 50 per cent, this state of incomplete differentiation appears to be common and it may even occur in all clones at an early stage. Such a conclusion is reinforced by preliminary observations (Allen and Nanney, unpublished) on multiple isolation series from single caryonides. A large majority of such series are found at maturity to contain either a few unstable sub-lines or pure sub-lines of different types. Hence, the differentiation process may be divided arbitrarily into two phases—the initial nuclear restriction occurring during macronuclear development which often (and perhaps always) leaves two or more potentialities, and the terminal nuclear restriction in which the capacities of a given nucleus are fixed in one potentiality. Since it ap-

peared possible that the two phases of differentiation were essentially alike, attention has been focussed on the somewhat simpler terminal phase and the analysis to be presented deals primarily with this phase.

The present studies were initiated in order to characterize as fully as possible the properties of selfers—the mating type potentialities associated in such clones, the rates of fixation to the different types, and the variability within these clones—in short, the specific properties of the system with which any explanation must cope. Preliminary observations have appeared elsewhere (Allen, 1956; Allen and Schensted, 1957; Nanney, 1957a). A mathematical treatment of sub-nuclear segregation is presented by Schensted in an accompanying paper.

MATERIALS AND METHODS

The stocks used in this study were exclusively of the Family A genotype and were derived through inbreeding from a cross of WH-6 and WH-14. The cultures were grown on a Cerophyl infusion inoculated with *Aerobacter aerogenes* (abbreviated "culture fluid"). The details of the culture methods have been presented elsewhere (Nanney and Caughey, 1955).

GENERAL PROPERTIES OF SELFERS

When selfing caryonides are detected in the laboratory they are maintained by making multiple single cell isolations from the culture in which selfing is first observed. The multiple sub-lines are then continued by making single cell isolations daily. Since conjugation occurs only when the cultures are beginning to starve, and since starvation occurs only after two days growth in depression slides, the lines are perpetuated without an intervening sexual stage. In the course of following serial sub-cultures occasional depression cultures will fail to conjugate. Rarely the next sub-culture in the series will again self, but in most instances the loss of selfing ability is permanent and all successive sub-cultures are pure for one mating type. In no instance have three non-selfers in a series been followed by a selfer; hence, the operational definition of stabilization (loss of capacity to self) is the failure to observe selfing pairs in three successive sub-cultures.

The general properties of selfers of Family A can be summarized as follows:

(1) *Most selfers studied at maturity have dual potentialities.* A study of 64 independently derived selfers revealed 52 which stabilized at two different types. From the remaining 12 selfers only one pure type was established. This failure to detect a second type is understandable in view of the properties of the I-VI selfer to be discussed below and should not be interpreted as indicating an absence of another potentiality. It is also possible that some of the dual systems would have produced still a third type if studied intensively.

(2) *Certain combinations of mating type potentialities occur in high frequency.* Thus, 37 of the 52 dual systems characterized had the potentiali-

ties for types I and VI; 9 were III-VI combinations; 4 were I-III combinations and one each were I-V and V-VI. Although mating type II was never characterized in combination with another type, 3 of the 12 incompletely analyzed systems contained the II potentiality associated with another undetermined type. Consideration of the significance of this distribution will be deferred.

PROPERTIES OF THE I-VI SELFER

Intraclonal variation

The most common dual system, I with VI, was studied intensively by a number of different methods. From the beginning, considerable intraclonal variation was apparent. Two characteristics of this variation can be cited:

(1) *The sub-clones can be classified into types.* Pure cultures of both mating types are almost never produced from a small culture (8 fissions) when multiple isolations (30 to 60) are made into culture fluid before it is allowed to starve. By making multiple isolations different sub-clones may be characterized by their pattern of response. Three types of sub-clones could be distinguished: (a) either all the cells in a sub-clone gave rise to selfing cultures, or (b) some gave rise to selfing cultures and some to type I cultures, or (c) some gave rise to selfing cultures and some to type VI cultures. Further differentiation of the type of sub-clone giving rise to 100 per cent selfing cultures could be effected by starvation. For producing this effect, two series of single cell isolations were made from a number of sub-clones, one into distilled water and the other into culture fluid. The distilled water series was retransferred 24 hours later into culture fluid. In addition to the types of sub-clones previously recognized, two types appeared in the distilled water series. These were sub-clones from which I (or VI) stable derivatives appeared only after starvation and not in the culture fluid series.

(2) *The sub-clonal types tend to be perpetuated in vegetative reproduction, but eventually all types of sub-clones can be derived from any original type.* When sub-clones derived from a common ancestor 20 fissions earlier were compared, they were alike qualitatively as well as quantitatively. In contrast, those removed by 120 fissions from a common ancestor showed differences. Both pure mating types were represented and different degrees of stabilization were observed. This implies that the nuclear condition of a particular type of sub-clone is not exactly reproduced at a given fission, but that the deviations from the initial state are so small that they must be accumulated over many fissions before they can be revealed. Thus, no one sub-clone directly manifests all the potentialities available but these potentialities can be detected through sampling many sub-cultures.

Time dependent changes in the system

(1) *The capacity of a sub-clone to produce a given pure type is inversely related to its capacity to produce the alternative type.* This conclusion is

suggested by the data cited above, but is shown more quantitatively by experiments in which a sub-clone is characterized not only by the immediate behavior of the cells isolated from it, but by the subsequent behavior of these isolates when maintained in daily transfer series. When multiple isolations are made from a sub-clone, some may produce stable cultures immediately but others will give rise to stable cultures only after many transfers. When the cumulative frequencies of the pure types are plotted as a function of fissions, graphs of the sort shown in figure 1 are obtained.

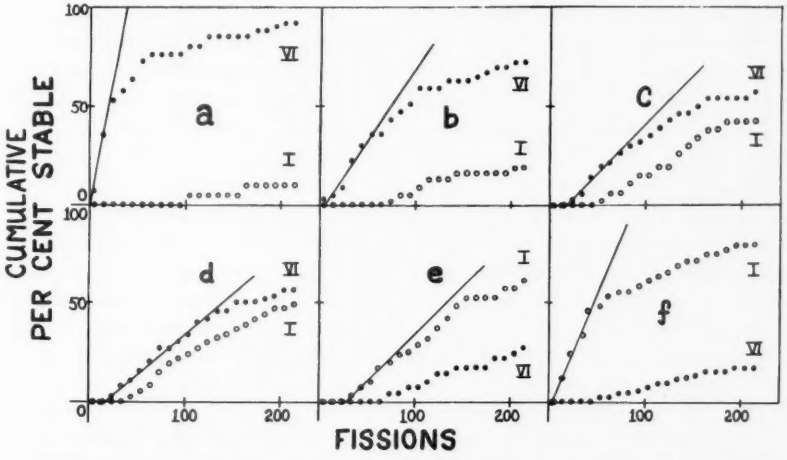

FIGURE 1. Cumulative frequencies of stabilization to mating types VI (solid circles) and I (open circles) as a function of fissions in selected sub-clones of I-VI selfers.

Some sub-clones (a) produce type VI at a high initial rate; these sub-clones produce type I then only after a delay and at a slow rate. Other sub-clones (f) produce I at a high initial rate and produce VI at a low rate and after a delay of a number of fissions. Those sub-clones that show a lower rate for the predominant type (b, c, d, e) show a somewhat higher rate for the secondary type. These relationships appear on the basis of the available information to be completely reciprocal. They imply a more or less continuous spectrum of sub-clones possible within a selfer, from those with one potentiality being highly predominant, through those with more or less equal potentialities to the two types, to those in which the other type is predominant.

(2) *When multiple isolations are initiated from a particular sub-clone, the initial rates of stabilization to the two types may be very diverse, but eventually the rates of stabilization to the two types in the remaining selfing lines approach equality.* The above experiments suggested a gradual change in the rates of stabilization to the two types, but the sample sizes were too small to permit an adequate description of these changes. For this pur-

pose a large scale experiment was inaugurated as follows: a single cell from a I-VI selfer was isolated, and after it had undergone six fissions, 20 single cell isolations were made and allowed to divide six more fissions. From each of these 20 sub-cultures 60 further isolations were made, yielding a total of 1200 sub-clones of two degrees of relationship. These subclones were perpetuated by daily transfer of single cells from growing cultures until stable. When stable, they were tubed and tested for mating type.

The fraction of stable cultures arising within a given fission interval divided by the total number of isolations from selfing cultures is defined as the "frequency of new stable cultures." The "rate" of production of new stable cultures per cell division is approximated by dividing the frequency of new stable cultures by the number of cell divisions. Certain complications arose in making these calculations, the most serious being the lack of uniform growth rates in the various isolation series. Certain cell lineages became defective for reasons not yet entirely clear and the growth rate in these lineages declined. For this reason it was necessary to record the growth rate of all lineages and to score a stabilization in terms of fissions rather than time. The frequencies of stabilization were calculated for each five-fission interval and then converted to rates per cell division by dividing by five. As the experiment continued, the fraction of selfing lineages declined—both through death in the defective lines and stabilization —and the rate of stabilization had to be averaged for longer and longer intervals in order that adequate numbers could be sampled.

A condensed summary of the results of this experiment is given in table 1 and figure 2. It shows first a rapid rise in the rate of stabilization of the predominant type (VI), followed by a gradual decline extending over a long period of time. Secondly, it shows a progressive increase in the rate of stabilization of the minority type (I), from zero to approximately the level finally reached by the predominant type. Thus, in spite of the initial disparity, the rates of stabilization to the two types seem to reach an identical equilibrium value—or "equivalence." The precise number of fissions required to reach equivalence is difficult to determine, since the number of lines remaining after a hundred fissions is too small to provide an adequate sample, even when the largest initial sample size capable of being handled is employed. This difficulty was in part circumvented in later experiments by periodically expanding the remaining selfers into multiple lineages. In this experiment, equivalence was probably not reached earlier than 100 fissions or later than 200 fissions.

In contrast to the gradual approach to equivalence, the total rate of stabilization (the *sum* of the two rates of stabilization) reaches an early plateau and is maintained thereafter with only random fluctuations. The plateau or equilibrium value reached by the total rate is 0.0113 per fission, and in this experiment this value was reached by about 25 fissions. All further changes in the rates of the two types are strictly complementary. The characteristic noted under (1) is therefore reinforced by this observation concerning the behavior of multiple isolations from a single sub-clone.

TABLE 1

THE PROBABILITY (P) THAT A CELL ISOLATED FROM A I-VI SELFING CULTURE WILL BE FIXED AFTER f FISSIONS AND THE RATE OF STABILIZATION TO VI AND TO I PER FISSION (P/5) AFTER f FISSIONS. THE TOTAL RATE EQUALS THE SUM OF THE TWO RATES: (P/5) VI + (P/5) I

	VI		I		Total Rate
f	P	P/5	P	P/5	(P/5) VI + (P/5) I
0–12	34/1206	.00470*	0/1206	0	.00470*
13–37	271/4691	.01155	2/4691	.000086	.01164
38–62	170/2962	.01148	5/2962	.00034	.01182
63–87	73/1915	.00762	15/1915	.00157	.00919
88–112	52/1247	.00834	18/1247	.00289	.01123
113–137	31/762	.00814	11/762	.00289	.01103
138–162	19/516	.00736	10/516	.00388	.01124
163–212	20/600	.00667	19/600	.00633	.01300
213–262	6/269	.00446	8/269	.00595	.01041
262+	6/319	.00376	13/319	.00815	.01191

*P/6.

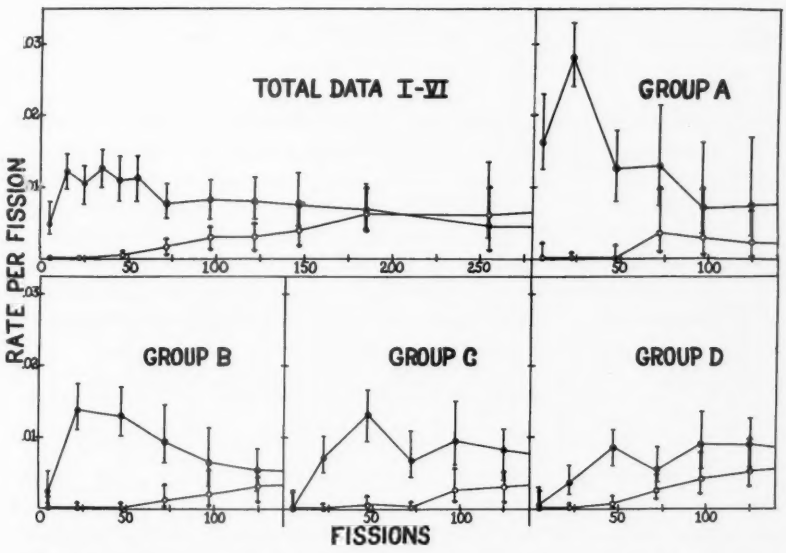

FIGURE 2. Time course of stabilization rates per fission to mating types VI (solid circles) and I (open circles) for 1200 sub-clones (total data) and for groups A, B, C, and D of the I-VI selfer. Vertical bars represent the 95 per cent confidence limits.

(3) *Peaks in the rate of production of the predominant type vary in height and time among related sub-clones.* The 1200 sub-clones of the experiment under discussion consisted of 20 "pedigrees" of 60 sub-clones each. Although significant heterogeneity was noted within the 20 pedigrees as to their initial rates to the predominant type (VI), the rates may be ranked to

TABLE 2

RATES OF STABILIZATION (P/5) TO VI AND TO I PER FISSION OF GROUPS A, B, C AND D AFTER f FISSIONS

	A		B		C		D	
f	P	P/5	P	P/5	P	P/5	P	P/5
				VI				
0–12	29/299	.01617*	4/300	.00222*	0/306	0	1/304	.00055*
13–37	118/843	.02800	82/1194	.01374	48/1337	.00718	23/1317	.00349
38–62	23/365	.01260	48/748	.01283	57/866	.01316	42/983	.00855
63–87	14/216	.01296	23/494	.00931	19/567	.00670	17/638	.00533
88–112	5/141	.00709	10/314	.00637	19/396	.00960	18/396	.00909
113–212	9/196	.00918	14/548	.00511	22/569	.00773	25/565	.00885
213+	2/110	.00364	4/203	.00394	4/197	.00406	2/78	.00513
				I				
0–12	0/299	0	0/300	0	0/306	0	0/304	0
13–37	0/843	0	1/1194	.00017	1/1337	.00015	0/1317	0
38–62	0/365	0	0/748	0	2/866	.00046	3/983	.00061
63–87	4/216	.00370	3/494	.00121	0/567	0	8/638	.00251
88–112	2/141	.00284	3/314	.00191	5/396	.00253	8/396	.00404
113–212	2/196	.00204	11/548	.00401	10/569	.00351	17/565	.00602
213+	1/110	.00182	7/203	.00690	10/197	.01015	3/78	.00769

*P/6.

form a continuous series. Then, by arbitrarily lumping segments of the continuous series, four groups (A, B, C, D) of five pedigrees each were formed. In this manner the sample sizes were improved and comparisons among the groups could be made. The results of this analysis are summarized in table 2 and figure 2. The important feature to be noted is that each group has a unique peak rate to VI, and the height and time of the peak rate is characteristic of each group. It is highest and earliest in group A and lower and later in groups B, C, and D, in order. The significance of this observation will become apparent in the following section.

ANALYSIS OF NUCLEAR SEGREGATION

Model of nuclear segregation

An interpretation of nuclear stabilization in the selfers of *P. aurelia* (Nanney, 1953) was based on Sonneborn's hypothesis (1947) of macronuclear sub-units. Indeed, the model to be proposed bears many similarities to the one Sonneborn proposed for the segregation of mutated sub-units and to the one developed by Kimball and Householder (1954) to account for segregation of radiation induced defects. If in selfers such sub-nuclei were differentiated as to mating type potency, the stabilization of pure types could result from the fixation of pure macronuclei through segregation (figure 3). The data presented in the previous section permit the first quantitative evaluation of this hypothesis.

The qualitative characteristics of the model will become quickly apparent. The probability of forming a nucleus containing only one type of sub-

nucleus at a particular cell division would depend on the total number of sub-nuclei in the parent cell and the relative numbers of the different types (e.g., the ratio of type A to type B). In general, the probability of "stabilization" would be inversely related to the total number of sub-nuclei (N)—high when N is low and low when N is high—while the probability of stabilizing a *particular* mating type would depend upon the frequency of the corresponding type of sub-nucleus. Should less than half of the units in the parent cell be of a particular type, then the probability of a pure daughter cell of that type would be zero.

When the mean number of sub-nuclei of a certain type in a population of related cells is high, the rate of stabilization to that type will also be high, and the rate of stabilization to the other type (or types) will be necessarily low. However, with selective removal of those cells which stabilize during growth, sub-nuclei of the predominant type are also selectively removed and the mean frequency of that type in the population will fall. As a result of this procedure, among the remaining cells the rate of stabilization to the predominant type will fall progressively in time while the rate of stabilization to the minority type will rise until the two rates approach each other asymptotically.

The observations reported in previous sections are in essential agreement with these considerations, but the quantitative features of the model require further exploration. Formulation of the model in precise terms has been accomplished by Schensted and is presented in an accompanying paper.

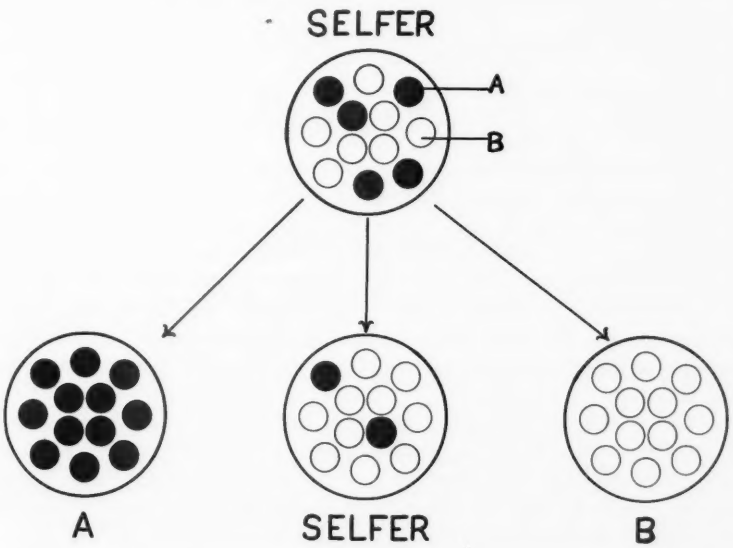

FIGURE 3. Schematic representation of nuclear segregation. The macronucleus of a selfer contains a mixture of two types of sub-nuclei, A (solid circles) and B (open circles). With segregation, macronuclei emerge pure for A or pure for B.

In developing this model certain simplifying assumptions about the behavior of sub-nuclei during their replication and segregation to daughter cells have been adopted:

(1) Each sub-nucleus replicates once during the interfission cycle.
(2) The total number of sub-nuclei at the time of division remains constant.
(3) Equal numbers of sub-nuclei go to each daughter cell.
(4) During division the segregation of the sub-nuclei occurs completely at random and independent of their quality; hence, for one of the daughter cells any sampling of sub-nuclei is possible; the other daughter cell is completely determined as to its share of sub-nuclei.

Since any or all of the above assumptions may be incorrect, the *direction* of possible bias should be pointed out. In general, deviations from the assumptions would result in greater variability in the distribution of sub-nuclei. For example, should daughter sub-nuclei tend to remain together geographically, assumption (4) would be violated and greater deviation between daughter cells in their macronuclear complement would result. Under conditions of greater variability, the probability of stabilization would be greater, given a certain number of sub-nuclei, and in estimating the number of sub-nuclei from the rate of stabilization, this number would tend to be too low. However, the *extent* of the underestimate cannot be easily determined.

The two parameters selected for deriving the theoretical curves were (1) the total rate of stabilization at equilibrium (r), and (2) the input ratio of the two types. Schensted (1958) shows that $r = \frac{1}{2N - 1}$ where N = the number of sub-nuclei in a daughter cell. Since r can be reliably measured and the formula $r = \frac{1}{2N - 1}$ is sensitive to changes in r, the approximate value of N may be estimated rather accurately. For example, if r = 0.0101, N = 50; but, if r = 0.00503, N = 100. Applying the formula to the data reported, r = 0.0113 and N = 45.

The input ratio varies from experiment to experiment and is expressed in terms of the parameter k, the frequency of the minority type. Estimation of k is made possible by the property of the model which requires that the cumulative frequencies of pure sub-cultures correspond to the initial frequencies of sub-nuclear types when all the sub-clones are transferred until they stabilize.

Utilizing only these two parameters, N with different values of k, a set of theoretical curves may be generated (see Schensted, 1958). The applicability of the model then rests upon the agreement between the theoretical and experimental curves. Specifically, the model yields the following parameters independently of the experiments:

(1) The time to equilibrium in the total rate.
(2) The height of the peak rate of the predominant type for any given value of k.

(3) The time of the peak rate of the predominant type.
(4) The time required to reach equivalence in the rates to the two types.

The degree to which these parameters find agreement in the theoretical and experimental curves provides a test of the hypothesis of nuclear segregation.

Application of the model

The set of theoretical curves for various values of k when N = 45 may be found in the accompanying paper by Schensted (1958). An experimental estimate of k may be obtained from the ratio of the stable cultures of each type accumulated at the end of the experiment. For example, the sub-clones of the I–VI selfer yielded 101 I : 682 VI. The fraction of the minority type (I), 101/783, is then multiplied by N and results in a k value of 6. Hence, we arrive at an input ratio of 39 VI : 6 I for this experiment. For the four groups A, B, C, and D, the following input ratios were estimated: A—43 : 2; B—39 : 6; C—38 : 7; D—34 : 11.

These calculations, however, all tend to underestimate k for the following reason. During the course of the experiment many of the sub-clones died. Since the sample size was larger at the beginning of the experiment, those sub-clones which stabilized early contributed a disproportionate number of the predominant type, and the frequency of late stabilizations (more often to the minority type) was diminished. Thus, in the final ratio of the two stable types the predominant type was favored at the expense of the minority type. The point to be made here is that the theoretical curves used to compare with the experimental curves actually have k values somewhat higher than

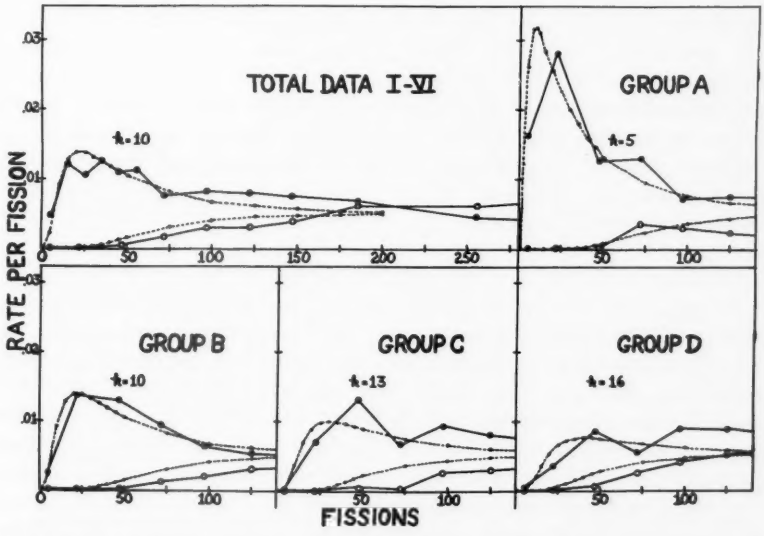

FIGURE 4. Comparison of experimental curves (solid lines) and theoretical curves (broken lines) for 1200 sub-clones (total data) and for groups A, B, C, and D of the I–VI selfer. The k value of each set of theoretical curves is indicated.

the estimated ($k + 3$ to $k + 6$). These curves give reasonable agreement with each other (figure 4).

A rigorous test of the hypothesis of nuclear segregation lies in the extent to which the theoretical and experimental curves fit. An exact fit is exexpected. When a Chi square test is performed, significant deviations appear in portions of the data. However, these deviations may be attributed to two sources: to the death in the experiment and to the design of the experiment. Since only 20 cells were sampled at six fissions—and the resulting populations expanded to 60 lineages each, deviations from randomness could have been accentuated—and the average of the data may not represent the true mean for a particular observation. Using the Chi square test, good fits of the experimental and theoretical curves are obtained for groups A and B but not for groups C and D. When the total data are then analyzed, Chi square equals 35.4, and with 19 degrees of freedom, $p = .01$. When Chi square is partitioned, the greatest source of error is contributed by the I curves ($p < .01$); the fit of the VI curves is good ($p = .2$). In later experiments where death and experimental design presented no problem in the analysis, excellent agreement between the theoretical and experimental curves was achieved using the Chi square test.

A less rigorous but adequate test of the hypothesis of nuclear segregation consists of an examination of the correspondence between the predicted values for various parameters and the experimental observations. Such a comparison is illustrated in table 3. Good agreement is found in the time to equilibrium of the total rates and in the height of the peak rates to VI. Some displacement to the right may be noticed in the time of the peak rates in the experimental curves of the four groups as compared to the theoretical curves. Least accurate is the measure of the time to equivalence in the rates of the two types. This is the most difficult of the four parameters to measure experimentally because deviations from randomness may be expected in the selection of the remaining selfers. Nevertheless, within the limits of the data obtained, the hypothesis of nuclear segregation is supported because of the agreement between the observed and expected results for the four parameters—and in the close but not exact fit of the experimental and theoretical curves.

Predictions of the model

In addition to predicting the time course of stabilization in I-VI selfing clones, the hypothesis of nuclear segregation generates several other predictions concerning selfers and their behavior under certain experimental conditions, which are capable of experimental verification. The first of these involves the stabilization characteristics of dual systems other than the I-VI combination. The frequency of this particular combination among mature selfers might be interpreted as indicating that it is much less likely to "stabilize" than other combinations and that these other combinations have different stabilization characteristics. If this inference were correct, then different selfers would have to be interpreted as possessing different

TABLE 3

COMPARISON OF THEORETICAL AND EXPERIMENTAL CURVES IN FOUR PARAMETERS

	Total Data		Group A		Group B		Group C		Group D	
	Theor. k = 10	Exp.	Theor. k = 5	Exp.	Theor. k = 10	Exp.	Theor. k = 13	Exp.	Theor. k = 16	Exp.
Parameter:										
(1) Time to equilibrium in total rate	40–50	13–62	50–75	38–62	40–50	13–62	40–50	38–62	45–50	38–62
(2) Height of peak (VI)	.0138	.01155	.0317	.02800	.0138	.01374	.00991	.01316	.00757	.00855–.00909
(3) Time of peak (VI)	22	13–37	10	13–37	22	13–37	30	38–62	40	38–112
(4) Time to equivalence in two rates	100–200	138+	125–200	63+	100–200	88+	100–200	113+	100–200	63+

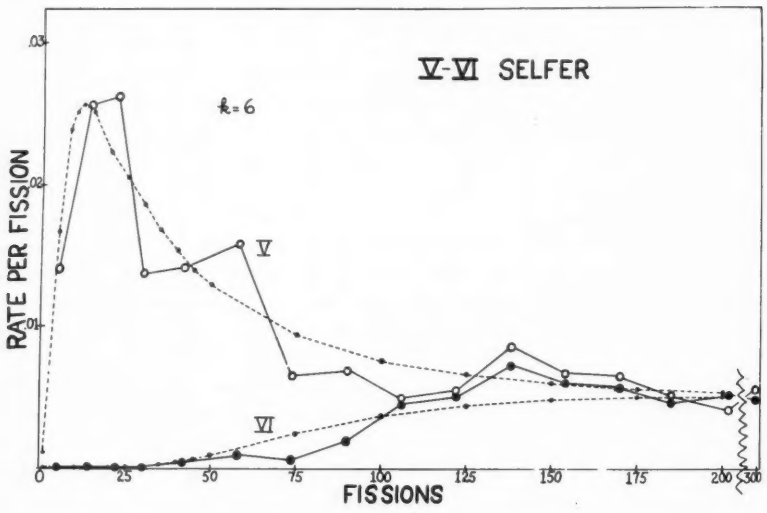

FIGURE 5. Comparison of experimental curves (solid lines) and theoretical curves (broken lines; k = 6) for sub-clones of the V–VI selfer. Open circles represent mating type V, solid circles mating type VI.

numbers of sub-nuclei or as varying in some other significant characteristic of the model. However, if the model had to be reconstructed in order to fit each individual combination, its support would be greatly weakened. On the other hand, the different observed frequencies of the various dual systems could reflect differences in their probability of origin, rather than in their characteristics once established. If this were the situation, then regardless of genotype or of the particular mating type potentialities associated in a dual system, the kinetics of stabilization should be the same; and reliance upon the model would be greatly strengthened.

Thus far only one additional system has been carefully studied, a V–VI selfer of Family A genotype, and a system which is observed less than a twentieth as often as the I–VI selfer. That the kinetics of stabilization are the same as in the I–VI selfer is illustrated in figure 5. Here, the total rate of stabilization again fluctuates around 0.0113 after an initial peak and the rates to the two types reach equivalence by 75 to 100 fissions. The establishment of equivalence is better documented than it was for the I–VI selfer. This was made possible by expanding the lineages of the remaining selfers duri..g the later fission intervals. The theoretical curve which fits best the experimental curve has a k value of 6, in agreement with the estimate obtained directly from the data (V = 369; VI = 51). This experiment was relatively free from death of the sub-clones so that no adjustment of k was needed such as that required in the previous experiment. In this experiment the theoretical and experimental curves show a good fit as determined by the Chi square test (p = .5).

A second test of the model concerns the effect of temperature on the stabilization characteristics. One of the chief alternatives to the segregation hypothesis is based on the concept of steady-states (Nanney 1957a) in which nuclear attributes are determined by biochemical antagonisms. Should this type of mechanism be operating, tne rates of stabilization might be expected to be influenced by the temperature at which the cells are grown. On the other hand, under a mechanism such as nuclear segregation, the pattern of sub-nuclear segregation would scarcely be expected to be altered by the temperature, provided the rate of stabilization were calculated on a per fission basis. The chief restriction to this expectation is that the total number of sub-nuclei (and hence DNA) must remain constant during growth at different temperatures. If, under certain conditions, the DNA content were low, the stabilization rate should be correspondingly high. Information on this point is lacking, but preliminary studies on stabilization rates at different temperatures have been carried out (figure 6) with no apparent effect of temperature observed. Moreover, exploratory studies on the effects of various physical agents (heat and cold shocks, centrifugation, ultraviolet irradiation) have revealed no effect on stabilization. These data may thus provide further support for the model.

A third test of the model concerns the effect of starvation on the stabilization characteristics, for if the segregation model is correct, it must pro-

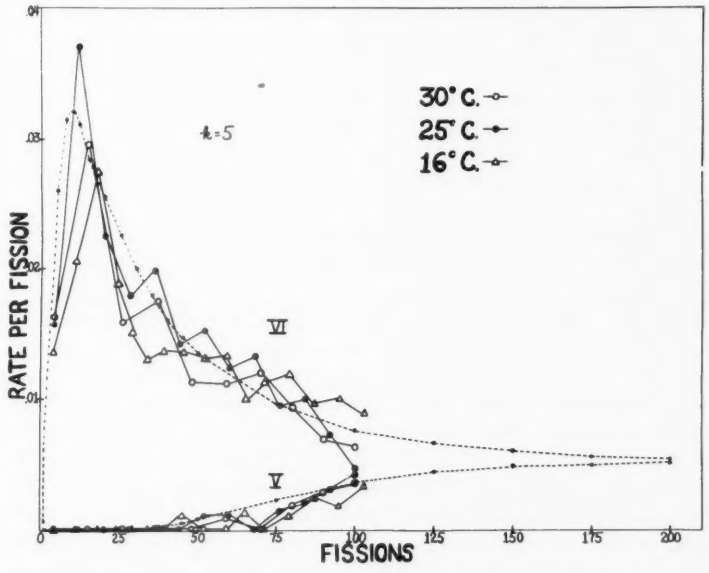

FIGURE 6. Comparison of stabilization rates of sub-clones of the V-VI selfer grown at three different temperatures (16°C, 25°C, 30°C). Experimental curves (solid lines) may also be compared with theoretical curves (broken lines) with a k value of 5.

vide some explanation for this phenomenon. Starvation of cells from selfing cultures usually results in the formation of additional stable cells (Nanney and Caughey, 1955). Initially this was interpreted as rendering improbable the segregation hypothesis. However, the phenomenon may be reconciled with the model by assuming that the number of sub-nuclei is reduced upon starvation. If these were reduced at random, the probability for a pure nucleus is increased. The validity of this interpretation, however, has not been investigated systematically. Moreover, still further complications must be mentioned: the I-VI and V-VI selfing clones, which have been studied most, differ in their response to starvation.

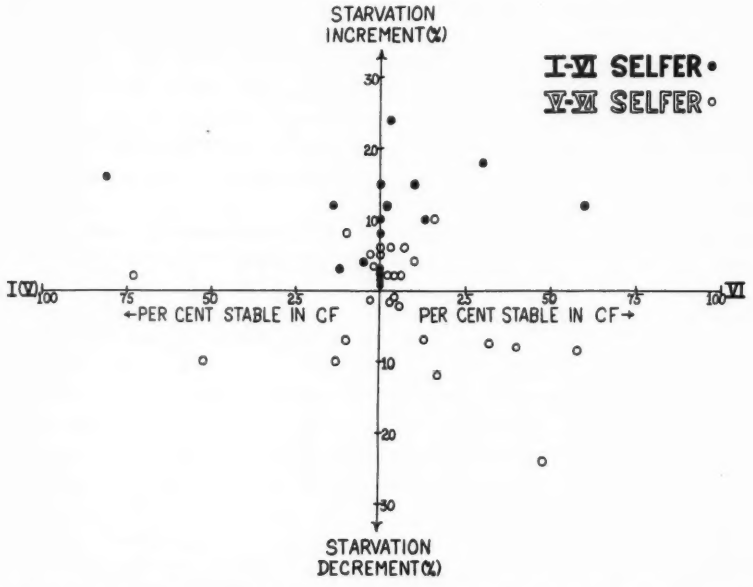

FIGURE 7. Comparison of effect of starvation on I-VI and V-VI selfers. 30–60 isolations into culture fluid and into distilled water were made for each sub-clone (see text).

This difference is demonstrated best by a scatter diagram (figure 7). Along the horizontal axis are represented the various frequencies of stabilization in culture fluid ("CF") to the two types concerned for different sub-clones, starting at a central point ("O" stable) and going to the left or to the right of this point for a given type. Along the vertical axis are represented the starvation increments and decrements. Increments may be found on the top portion of the graph, decrements on the lower portion of the graph. Sub-clones of the I-VI selfer are scattered on the top portion of the diagram, while those of the V-VI selfer are randomly scattered. Thus, the I-VI sub-clones tend to stabilize when starved, but the V-VI sub-clones do

not. This difference is difficult to explain on the basis of the model. However, only one clone was intensively studied for each type of selfer; hence, this difference in response to starvation may reside in other properties of the two clones than in their possession of different combinations of mating type potentialities. This explanation does not appear unreasonable in view of a recent report (McCashland and Johnson, 1957) on macronuclear volume in amicronucleate strains of Tetrahymena. About half of the strains showed no changes throughout the entire growth cycle, while the other half showed a reduced nuclear volume during the lag phase of growth. Clearly such studies must be extended to these selfing clones, since the starvation effect is the chief challenge to the segregation model at the present time.

Finally, the proposed model yields an estimate of 45 sub-nuclei in a newly formed daughter cell, or 90 sub-nuclei in a cell just prior to division. As pointed out above, this estimate may tend to be low. Hence, the number of sub-nuclei in a newly divided cell may be 50 or greater. Since the sub-nuclei are conceived as being diploid, like the micronucleus, the ploidy level of the macronucleus should vary from 100 to 200, depending upon the stage of the mitotic cycle, and the DNA content of the macronucleus should vary from 50 to 100 times that of the micronucleus. Volume measurements on fixed cells are consistent with this expectation, but they are not completely reliable. More precise tests need to be undertaken.

JUSTIFICATION OF THE NUCLEAR SEGREGATION HYPOTHESIS

Although the emphasis is being placed on the nuclear segregation hypothesis as an explanation for the foregoing results, other mechanisms certainly cannot be excluded at this time. In particular, a mechanism based upon alternative steady-states may be applicable. However, such a hypothesis is much more difficult to test than a particulate hypothesis. Hence, in this section, the evidence for and against the nuclear segregation hypothesis will be stressed.

Beyond the application of the model of nuclear segregation to the data reported above, the model itself and the considerations involved in deriving the result, $r = \frac{1}{2N-1}$ (Schensted, 1958) should prove useful in other situations where a particulate hypothesis is pertinent. Here, however, the question which needs examination is whether nuclear segregation is a reasonable hypothesis on the basis of what is known about the structure of the ciliate macronucleus.

The central problem lies in the reconciliation of two different kinds of evidence—on the one hand, cytological—and on the other, genetic. Neither conventional cytological procedures nor electron microscopy gives evidence of a sub-nuclear structure for Tetrahymena or its close relatives (Dippell, unpublished; Kimball, 1953; Sonneborn, 1949). Even Grell's observations (1956) of bundles of chromosomes (or chromosome complexes) produced by repeated endomitoses in the macronucleus of distantly related forms do not obviously support the hypothesis. Because of the lack of direct morpho-

logical evidence of sub-nuclei, the indirect evidence must be correspondingly strong if the segregation hypothesis is to be supported.

This evidence is derived primarily from observations on *P. aurelia.* In this species the macronucleus breaks down into 30 to 40 fragments at the time of nuclear reorganization. Under appropriate treatment (Sonneborn, 1947) these fragments may regenerate and a single fragment may reconstitute an entire macronucleus, which in all cases examined controls the same hereditary traits as the whole nucleus. Each fragment must, therefore, contain a balanced set of genetic determiners. Since the fragments maintained the original genetic constitution, even when the original nucleus was heterozygous for genetic markers, Sonneborn concluded that they contained at least one diploid set of chromosomes. Such results would be expected under either of two hypotheses, the sub-nuclear hypothesis of Sonneborn, or the "grab-bag" hypothesis of Kimball (1943). The chief distinction between the two lies in the organization of the chromosomes in the macronucleus: either they are organized as discrete diploid genomes or they are randomly distributed as single chromosomes. The total ploidy level required in the sub-nuclear interpretation is only about 80; that required for the "grab-bag" interpretation—many hundreds. No direct estimate of the ploidy level in *P. aurelia* is available, but in *P. caudatum* Moses (1950) calculated that the macronucleus possessed 40 times as much DNA as the micronucleus. However, in some strains of *P. caudatum* the micronucleus may also be polyploid. Such studies in *P. aurelia* are more difficult because of the type of macronucleus present: it is small and vesicular, and the chromatin is organized as a small, dense mass. Nevertheless, it seems clear that the ploidy level in some ciliate macronuclei is sufficiently low to render a random assortment of chromosomes improbable. Unfortunately both cytological and genetic information in the same species are not available.

The second genetic argument for the subnuclear structure is an extension of the first. If the chromosomes are distributed at random during nuclear division, nuclei more distantly removed from their origin would tend to become progressively less balanced in their genetic constitution. Faure-Fremiet (1953) has invoked this consideration to explain "ageing" in the ciliates. This mechanism would be directly demonstrated should homozygotes be derived from heterozygotes during vegetative growth. Although inviability may set in before the test becomes adequate, attempts at such derivation in "aged" cultures have met with no success (Sonneborn, Schneller and Craig, 1956). Using the most conservative assumptions Sonneborn (unpublished) has estimated that a total ploidy level of at least 320 would be required to be consistent with the "grab-bag" interpretation. In short, some mechanism for maintaining balanced chromosome sets is probably operating in the macronucleus of *P. aurelia*, but morphological evidence for such a mechanism is as yet absent.

Other genetic evidence comes from the properties of selfers in *P. aurelia.* For example, the selfers of variety 1 studied by Kimball (1939) and of variety 4 studied by Nanney (1957 b) share many properties common to those

of Tetrahymena. In the latter case, the phenotype of a selfer could be explained as a consequence of the ratios of two kinds of sub-nuclei.

Evidence specifically supporting an hypothesis of macronuclear heterogeneity for selfers is also available in *P. aurelia.* Nanney (1953, 1954) has shown that fusion of macronuclei of independent origin can produce selfers even when selfers had not previously been found in the stock. These "artificial" selfers behaved in all respects like the spontaneous selfers in other stocks: they remained "unstable" for long periods of growth, but occasionally they gave rise to sub-clones pure for a mating type. Moreover, at macronuclear regeneration, selfing clones in *P. aurelia* yield much higher frequencies of pure types than in vegetative growth. All of these observations are thus compatible with the notion that mating type instability in this form is the result of macronuclear heterogeneity.

Before leaving selfers, mention should be made of the selfers which do not conform to the pattern observed above in Tetrahymena. In the Spirotrichs lack of mating type differentiation may be common and thus account for selfers (Kimball, 1943). In the Holotrichs at least two additional types of selfers may be found. Some selfers give rise to no pure mating types (Elliott and Nanney, 1952). Other selfers arise late in the life-cycle from pure cells by a process called "self-differentiation" (Jennings, 1941). From these selfers cells of pure mating type could be derived. Recently, Sonneborn (1957) has discovered strains in variety 15 of *P. aurelia* which show a diurnal alteration of mating types. Within a clone *both* mating types are expressed at different times, and, because of a lack of synchrony in the "switch-over" time, conjugation within the clone is observed.

NUCLEAR DIFFERENTIATION IN TETRAHYMENA

Since nuclear differentiation in the selfers of Tetrahymena is probably the result of a special mechanism, the focus of attention should be shifted to earlier stages of nuclear differentiation where the underlying mechanisms may have more general application. Here, the origins of sub-nuclear differences are of major concern. An understanding of the establishment of dual systems may provide some important hints about this process.

If all possible two-type associations could exist in Family A (I with II, I with III, I with V, etc.) a total of ten dual systems might be found. Actually only five such systems have been positively identified (I–III, I–V, I–VI, III–VI, and V–VI) although other systems were probably present (II–?). From Family B selfers three and even four different pure types were occasionally extracted (Nanney and Caughey, 1955). Certainly the establishment of multiple systems appears less common in Family A than in Family B.

When mature cultures are produced by serial isolation lines from recently derived caryonides, the majority are pure for some particular mating type. The relative frequencies of these types in any cross can be scored and are found to be reasonably constant for a particular genotype. The existence of dual systems at low frequency among mature clones raises several problems in interpreting the frequencies of pure types. The observation of phenotypic

stabilization after maturity and evidence for a similar stabilization of potentialities prior to maturity (Nanney and Caughey, 1955) clearly demonstrate that many, and perhaps even all, caryonides initially possess two or more potentialities. The final frequencies of pure types are therefore the result of the establishment of diverse dual systems of potentialities and the segregation of pure types from these.

If the following assumptions were all true, then equal frequencies of the different pure types would be expected. These assumptions are: (1) all systems are initially dual; (2) all dual systems are established with equal probability; (3) all dual systems stabilize at the same rate; (4) both types in a dual system stabilize with equal ease. Since the frequencies of the pure types vary considerably from equality (from V = 0.075 to VI = 0.39) in Family A one or more of these assumptions must be incorrect.

Pertinent to the argument are the observations on the types present in Family A selfers at maturity. Not only are the frequencies of types derived from such systems not equal to each other but they are not equal to the frequencies in pure cultures at maturity (I = 0.22, II = 0.16, III = 0.155, V = 0.075, VI = 0.39). Thus, 64 selfers yielded 44 instances in which type I was present (0.38), 3 with II (0.026), 14 with III (0.12), 2 with V (0.017), and 53 with type VI (0.46). Moreover, the frequencies of the dual systems are far from equal and differ from the joint frequencies of the contained pure types. For example, the most common dual system, I-VI, occurred in 37 (0.71) of the 52 systems completely characterized, where 28 would be the expected number (0.54).

These results suggest that assumption (2) is false. All dual systems are *not* established with equal probability. Moreover, both qualitative and quantitative factors play a part. Thus, the establishment of dual systems depends not only upon the quality of the mating type potentialities contained but also upon the initial ratio of the two types among the sub-nuclei. The final ratio of pure types is a result of both these factors, and the persistent dual systems are those which have a high probability of origin and an input ratio close to 0.5. For example, mating type V is rarer in selfers than in pure types, suggesting infrequent existence in dual systems, but when present, existing in high ratio.

Support of these views comes from preliminary observations on multiple sub-caryonides. These are obtained approximately 5-6 fissions after separation of the caryonides, and each sub-caryonide is carried to maturity. Most of the caryonides showed one of two different dual systems and characteristic input ratios were found for each of these systems. The input ratio was either near equality or slightly shifted in favor of I in seven I-VI caryonides, while the ratio was consistently in favor of VI in all five III-VI caryonides analyzed.

Many questions concerning this early stage of nuclear differentiation remain for future investigation. A more detailed description of the interrelationship of mating type potentialities is needed not only for Family A but for other genotypes. Perhaps under other experimental conditions some answers

may be forthcoming on how different mating type potentialities become associated in dual systems and how the ratio of types is established. Certainly the area is rich for investigating problems of nuclear differentiation and perhaps for gaining some insight into mechanism.

SUMMARY AND CONCLUSIONS

A detailed quantitative description of the properties of certain unstable clones (selfers) of Family A, variety 1, of *T. pyriformis* is given. Briefly, dual associations of mating types are frequently found in these clones, and cells, pure for one of the two mating types, are produced. The rates of production of the two types of pure cells from unstable clones are initially dissimilar but become similar after a hundred or more fissions. The sum of the two rates, however, comes to an early equilibrium.

These properties are most easily interpreted on the assumption that segregation of stable and differentiated macronuclear elements (sub-nuclei) occurs during vegetative reproduction. The accompanying paper by Schensted provides the mathematical basis for evaluating this interpretation precisely. In general, the mathematical model and the experimental results are in excellent agreement. The total number of diploid sub-nuclei in a newly divided cell is estimated at 45.

In spite of the support for the hypothesis of nuclear segregation, and the absence of decisive evidence against it, alternative explanations have not been rigorously excluded, and indeed cannot be until other models capable of detailed examination have been developed. Nevertheless, the available evidence in support of the proposed model renders it improbable that further analysis of this sort will be fruitful in understanding the origin of the more significant nuclear alterations involved in the differentiation of mating types in the ciliates. Utilizing different techniques, preliminary studies are reported which are directed toward an analysis of the earlier and more fundamental aspects of nuclear differentiation.

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APPENDIX

MODEL OF SUBNUCLEAR SEGREGATION IN THE MACRONUCLEUS OF CILIATES*

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We shall in this section outline the mathematical treatment of a simple idealized model which may serve to explain the foregoing experimental results. We shall first make a brief statement of the nature of the model treated and then shall go on to a discussion of its mathematical formulation.

NATURE OF THE MODEL

We consider a homogeneous population of biological cells each of which contains N particles. These particles are of two types which we shall designate as type A and type B. Initially let us suppose that each cell contains k particles of type A and $N - k$ particles of type B. Prior to the division of each cell it is imagined that each of the N particles contained within the cell replicates itself so that each cell will then contain 2N particles, 2k of type A and $2N - 2k$ of type B. Upon division of each parent cell into two daughter cells it is assumed that the 2N particles contained within each parent will divide themselves equally amongst the two daughters. It is, however, assumed that the segregation of the 2N particles into the two groups of N each will be "perfectly random" with respect to the selection of particles from amongst the two types or within either type. By a "perfectly random" segregation we mean one in which all the possible partitions of the 2N particles into two groups of N have equal probabilities of occurrence. This assumption of randomness is a convenient one in view of our ignorance of the forces which might prevail during such a segregation process. Having made this assumption, it will follow that the daughter population may no longer be expected to be homogeneous with respect to the number of the particles of type A and type B contained within a single cell. Instead, finite fractions of daughter cells may be expected to have numbers of particles of type A ranging from 0 to 2k if $k \leq N/2$ or from $2k - N$ to N if $k \geq N/2$. During each subsequent division cycle the process of the replication of the N particles within a cell and the concomitant random segregation of the resulting 2N particles into two groups, one of which is retained in each new cell formed, is imagined to recur. One may then ask after α generations have passed what fraction of the cells may be expected to contain 0, 1, 2, etc. particles of type A?

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We shall below, using the apparatus of the theory of probabilities and the matrix algebra, formulate the problem in mathematical terms. In addition, we shall develop a simple relationship which in conjunction with the available experimental data allows us to determine the value of N appropriate to the model. Using this value of N, we have with the aid of the MIDAC computer calculated numerical values for the expected fractions of cells containing 0 and N particles of type A for initial values of k running from 1 to N – 1 as a function of the generation number, α. Plots of these numbers and a comparison with the experimental results appear in an accompanying paper by Allen and Nanney (1958).

MATHEMATICAL FORMULATION OF THE PROBLEM

If by $p_N(j, l)$ we designate the probability that a parent cell containing l particles of type A (and N – l particles of type B) will produce a daughter containing j particles of type A, then under the assumption of random segregation we have:

$$(1) \qquad p_N(j, l) = \frac{\binom{2l}{j}\binom{2N-2l}{N-j}}{\binom{2N}{N}} \qquad \begin{matrix} j = 0, 1, 2, \ldots, N \\ l = 0, 1, 2, \ldots, N \end{matrix}$$

where the symbol $\binom{\alpha}{\beta}$ denotes the binomial coefficient given more explicitly as:

$$\binom{\alpha}{\beta} = \frac{\alpha!}{(\alpha-\beta)!\,\beta!} \qquad \text{for } \beta \leq \alpha$$

$$= 0 \qquad \text{for } \beta > \alpha$$

If by $p_N^{\alpha}(j)$, we designate the fraction of cells expected to contain j particles of type A after α generations then the set of quantities $p_N^{\alpha}(j)$, j = 0, 1, 2, ... , N are related to the corresponding set of quantities for the previous generation by the formula:

$$(2) \qquad p_N^{\alpha}(j) = \sum_{l=0}^{N} p_N(j, l)\, p_N^{\alpha-1}(l)$$

It will be convenient to think of the N + 1 quantities $p_N^{\alpha}(j)$ as forming the components of an N + 1 dimensional vector. We shall designate this vector by the symbol p_N^{α}.

$$p_N^{\alpha} = \begin{bmatrix} p_N^{\alpha} & (0) \\ p_N^{\alpha} & (1) \\ \vdots & \\ p_N^{\alpha} & (N) \end{bmatrix}$$

Occasionally we shall drop the subscript N and refer to this vector as p^{α}. In addition it is convenient to consider the N + 1 by N + 1 matrix $\mathbf{P}_N$ which is formed by the elements $p_N(j, l)$ defined in equation (1), the index j designating the row and the index l designating the column of the corresponding element. Using this notation we may formally write the solution to our problem as:

$$p_N^{\alpha} = (\mathbf{P}_N)^{\alpha} p_N^0 \tag{3}$$

where $(\mathbf{P}_N)^{\alpha}$ designates the matrix $\mathbf{P}$ raised to the α power. The vector p_N^0 gives the fraction of cells which at the 0^{th} generation have $0, 1, 2, \ldots,$ N particles of type A. We shall in what follows consider the initial population to be homogeneous, each cell containing k particles of type A where k may be any integer from 0 to N. For such a population all the components of p_N^0 except its k^{th} will vanish and the k^{th} component will equal 1. As our labeling scheme starts from k = 0, we should explain that by the k^{th} component of $p_N^{\alpha}(j)$ we mean the component designated by j = k. This would be the $k + 1^{st}$ as one counts down. We shall designate by the symbol ${}^{k}p_N^{\alpha}$, the vector p_N^{α} corresponding to a 0^{th} generation of cells each of which contain k particles of type A. Equation (3) may be rewritten in terms of this notation as:

$${}^{k}p_N^{\alpha} = (\mathbf{P}_N)^{\alpha} \cdot {}^{k}p_N^0 \tag{3}'$$

From the form of ${}^{k}p_N^0$ we may conclude that the vector ${}^{k}p_N^{\alpha}$ will be given by the k^{th} column of $(\mathbf{P}_N)^{\alpha}$. Hence the matrix $(\mathbf{P}_N)^{\alpha}$ contains all the information of interest to us. Unfortunately the labor of evaluating $(\mathbf{P}_N)^{\alpha}$ for general N and α is immense. It is therefore most desirable to discover a simple criterion by means of which the appropriate value of N may be determined from the experimental results and then to concentrate upon the evaluation of $(\mathbf{P}_N)^{\alpha}$ for this value of N.

To this end we make use of the well-known theorem that the powers of a matrix may be expanded in terms of the powers of its eigenvalues, i.e., $(\mathbf{P}_N)^{\alpha}$ may be written in the form:

$$(\mathbf{P}_N)^{\alpha} = \sum_{\delta} \mathbf{C}_{\delta} \lambda_{\delta}^{\alpha} \tag{4}$$

(This expansion is valid here even though $\mathbf{P}_N$ does possess one doubly degenerate eigenvalue $\lambda = 1$. A matrix which possesses such a doubly degenerate eigenvalue might be expected to have in its expansion a term of the form $\mathbf{C}\alpha$. This can be shown to vanish for the present case). The matrices $\mathbf{C}_{\delta}$ are given by the relationship:

$$C_{\delta} = \begin{bmatrix} T_0^{-1}{}_{\delta} \\ T_1^{-1}{}_{\delta} \\ \vdots \\ T_N^{-1}{}_{\delta} \end{bmatrix} \qquad [T_{\delta 0} T_{\delta 1} \ldots\ldots T_{\delta N}] \tag{4}'$$

The matrix $\mathbf{T}$ the components of which appear in the above expression is the matrix which transforms $\mathbf{P}_N$ to its diagonal form, i.e.

$$(4'') \qquad \mathbf{T}\mathbf{P}_N\mathbf{T}^{-1} = \begin{bmatrix} \lambda_0 & 0 & 0 & 0 \\ 0 & \lambda_1 & & 0 \\ & & \ddots & \\ 0 & 0 & 0 & \lambda_N \end{bmatrix}$$

The λ's which appear in these equations are the eigenvalues of the matrix $\mathbf{P}_N$. The eigenvectors, χ_l, of the matrix $\mathbf{P}_N$ and the eigenvectors, y_j, of its transpose $\tilde{\mathbf{P}}_N$ are related to the matrices $\mathbf{T}$ and $\mathbf{T}^{-1}$ by the equations:

$$(4''') \qquad \chi_l = \begin{bmatrix} T_{0l}^{-1} \\ T_{1l}^{-1} \\ \vdots \\ T_{Nl}^{-1} \end{bmatrix} \qquad \begin{aligned} \tilde{y}_j &= [T_{j0}\ T_{j1} \ldots\ldots\ T_{jN}] \\ j &= 0,\ 1,\ \ldots\ldots,\ N \\ l &= 0,\ 1,\ \ldots\ldots,\ N \end{aligned}$$

From the nature of the foregoing expressions we see that a knowledge of the eigenvalues λ_α as well as the eigenvectors, χ_l and y_j, would immensely simplify the calculation of $(\mathbf{P}_N)^\alpha$. We have thus far been able to obtain the eigenvalues of the matrix $\mathbf{P}_N$ for all values of N but as yet have not obtained any simple expressions for χ_l and y_j. We will show below how a knowledge of the eigenvalues alone yields useful information and in particular allows us to determine the value of N easily from the experimental data.

Let us look more closely at $(\mathbf{P}_N)^\alpha$ in the light of equations (4) and in the light of some of the more obvious properties of the model. To begin with the quantities ${}^kp^\alpha(0)$ and ${}^kp^\alpha(N)$, $k = 0, 1, 2, \ldots, N$ form the first and last rows of $(\mathbf{P}_N)^\alpha$ respectively. They represent the fractions of the population which contain only particles of type B or only particles of type A. Hence these two fractions of the population will "breed true" and it may be expected that as $\alpha \to \infty$, ${}^kp^\alpha(0) + {}^kp^\alpha(N) \to 1$. Furthermore, since the ratio of the numbers of particles of the two types present in the population as a whole does not change ${}^kp^\alpha(0) \to 1 - \frac{k}{N}$ as $\alpha \to \infty$ and ${}^kp^\alpha(N) \to \frac{k}{N}$ as $\alpha \to \infty$. Hence the limiting form of $(\mathbf{P}_N)^\alpha$ will be:

$$(5) \qquad \mathbf{C}_0 = \begin{bmatrix} 1 & 1-\frac{1}{N} & 1-\frac{2}{N} \ldots\ldots 2/N & 1/N & 0 \\ 0 & & & & 0 \\ 0 & 0 & 0 & 0 & 0 \\ \vdots & & & & \vdots \\ 0 & 1/N & 2/N \ldots\ldots 1-\frac{2}{N} & 1-\frac{1}{N} & 1 \end{bmatrix}$$

Now it is obvious from the form of $\mathbf{P}_N$ (*vide supra* eq. 1) that it has two eigenvectors of the form:

$$\chi_0 = \begin{bmatrix} 1 \\ 0 \\ \vdots \\ \vdots \\ 0 \end{bmatrix} \quad \text{and} \quad \chi_1 = \begin{bmatrix} 0 \\ \vdots \\ \vdots \\ 0 \\ 1 \end{bmatrix}$$

corresponding to the eigenvalue $\lambda = 1$. As its other eigenvalues are necessarily less than 1 and, as we shall presently show, non-degenerate, the term in the expansion (4) of $(\mathbf{P}_N)^\alpha$ which corresponds to the eigenvalue $\lambda = 1$ must necessarily be $\mathbf{C}_0$ which is given by equation (5). Equation (4) may be rewritten as

$$(\mathbf{P}_N)^\alpha = \mathbf{C}_0 + \sum_{\lambda_\delta < 1} \mathbf{C}_\delta \lambda_\delta^\alpha \tag{6}$$

For large enough α the sum which appears on the right is dominated by the term corresponding the largest eigenvalue which is less than 1. We shall denote this eigenvalue by λ_L. For large α the expansion of $(\mathbf{P}_N)^\alpha$ takes the form:

$$(\mathbf{P}_N)^\alpha \cong \mathbf{C}_0 + \mathbf{C}_L(\lambda_L)^\alpha \tag{6}'$$

If we let ${}^kI^\alpha$ designate the sum of all the "impure" fractions, i.e.:

$${}^kI^\alpha \equiv \sum_{j=1}^{N-1} {}^kp^\alpha(j) \text{ or } {}^kI^\alpha \equiv 1 - {}^kp^\alpha(0) - {}^kp^\alpha(N) \tag{7}$$

then for large enough α, ${}^kI^\alpha \cong \sum_{j=1}^{N-1} C_{Ljk}(\lambda_L)^\alpha$.

The ratio $\dfrac{I^{\alpha-1} - I^\alpha}{I^{\alpha+1}}$ corresponds to what is termed the rate of stabilization r in the accompanying paper of Allen and Nanney (1958) and may be expressed, for α very large, as:

$$\frac{\sum_{j=1}^{N-1} C_{Ljk}(\lambda_L^{\alpha-1} - \lambda_L^\alpha)}{\sum_{j=1}^{N-1} C_{Ljk}\lambda_L^{\alpha-1}} = 1 - \lambda_L \tag{7}'$$

The above ratio is readily measurable experimentally and hence a determination of the analytical expression for λ_L, which will in general be a function of N, allows us to choose the value of N most suitable for use with the model. It should be noted here that the result in (7)′ is independent of k. This property is not one which is peculiar to the "random segregation" model. Any particle model which involves a definite probability matrix which relates the quantities $p^\alpha(j)$ to $p^{\alpha-1}(j)$ will have the property

that the limiting value of the ratio $\frac{I^{a-1} - kI^{a}}{kI^{a-1} - I}$ will be independent of k and equal to 1 minus the leading eigenvalue of the corresponding matrix.

We shall now turn to the derivation of explicit expressions for the eigenvalues λ_j of the "random segregation" matrix $\mathbf{P}_N$. The following identity suggested to us by A. Halpin and C. E. Schensted of the Willow Run Research Laboratory will be most useful in this connection:

$$(8) \qquad \sum_{j=0}^{N} \frac{\binom{2l}{j}\binom{2N-2l}{N-j}}{\binom{2N}{N}}\binom{j}{\mu} = \frac{\binom{2l}{\mu}\binom{N}{\mu}}{\binom{2N}{\mu}} \qquad \begin{matrix} l = 0, 1, \ldots, N \\ \mu = 0, 1, \ldots, N \end{matrix}$$

(The derivation of this result may be accomplished in the following manner. One expresses the left hand side of the equation in terms generalized factorials. Then one recognizes that the left hand side is related simply to the hypergeometric function, ${}_2F_1(\mu - N, \mu - 2l; N - 2l + \mu, 1)$, which may be expressed in terms of Γ functions through the relationship

$${}_2F_1(ab; c; 1) = \frac{\Gamma(c)\,\Gamma(c-a-b)}{\Gamma(c-a)\,\Gamma(c-b)}.)$$

The left hand side of equation (8) may be thought of as the matrix $\tilde{\mathbf{P}}_N$ operating on the vector whose components are $\binom{j}{\mu}$, $j = 0, 1, \ldots, N$. For each value of μ the components of this vector may be thought of as a polynomial in j of μ^{th} degree. The vector which has the components $\binom{2l}{\mu}$, $l = 0, 1, \ldots, N$ may also be thought of as a polynomial in l of degree μ. The fact that the result of operation by the matrix $\tilde{\mathbf{P}}_N$ on the vector $\binom{j}{\mu}$ yields a vector which is of no higher degree than μ leads us to the conclusion that the γ^{th} eigenvector of $\tilde{\mathbf{P}}_N$ may be expanded in terms of the vectors $\binom{j}{\mu}$ as follows $y_\gamma = \sum_{\mu=0}^{\gamma}\binom{j}{\mu}\beta_\mu$ where the β_μ's are constants. Explicit forms for the coefficients, β_μ, may be obtained by use of equation (8). We shall illustrate the procedure. First let us suppose that $\beta_\gamma = 1$. (This is no real restriction as y_γ times any constant is still an eigenvector of $\tilde{\mathbf{P}}_N$ corresponding to eigenvalue λ_γ). Substituting y_γ into the left hand side of equation (8), evaluating the right hand side of equation (8) and requiring that y_γ be the eigenvector of $\tilde{\mathbf{P}}_N$ corresponding to eigenvelue λ_γ, we obtain the following equation:

$$(9) \qquad \sum_{\mu=0}^{\gamma} \frac{\binom{2l}{\mu}\binom{N}{\mu}\beta_\mu}{\binom{2N}{\mu}} = \lambda_\gamma\left(\sum_{\mu=0}^{\gamma}\binom{l}{\mu}\beta_\mu\right)$$

In order to have the two sides of equation (9) equal we must match successively the various powers of 1 which appear. Matching the leading terms in 1^{γ} we get:

$$\lambda_{\gamma} = \frac{2^{\gamma}\binom{N}{\gamma}}{\binom{2N}{\gamma}} \qquad \gamma = 0, 1, 2, \ldots, N \tag{10}$$

Matching the terms in $1^{\gamma-1}$ we get an equation of the form:

$$\frac{-2^{\gamma-2}\binom{N}{\gamma}}{(\gamma-2)!\binom{2N}{\gamma}} + \frac{\beta_{\gamma-1}\binom{N}{\gamma-1}2^{\gamma-1}}{\binom{2N}{\gamma-1}(\gamma-1)!} = \frac{-\lambda\gamma}{2(\gamma-2)!} + \frac{\lambda\gamma\beta_{\gamma-1}}{(\gamma-1)!} \tag{10}'$$

From (10) we can find λ_{γ} and from (10)′ by substituting in the value of λ_{γ} obtained we can get $\beta_{\gamma-1}$. We can by continuing this procedure obtain all the β's. (For those interested in pursuing the problem of calculating the eigenvectors of $\mathbf{P}_N$ and $\tilde{\mathbf{P}}_N$, we make the following comments: By transforming $\tilde{\mathbf{P}}_N$ to a system in which the basis vectors are $\binom{j}{\mu}$ one may put $\tilde{\mathbf{P}}_N$ in triangular form. The matrices which accomplish this transformation are given by $S^{-1}_{j\mu} = \binom{j}{\mu}$, $S_{\mu 1} = (-1)^{\mu+1}\binom{\mu}{1}$. The resulting matrix has the form $0_{ik} = \frac{2^{2i-k}\binom{i}{k-i}\binom{N}{k}}{\binom{2N}{k}}$.) Since the equations become longer and messier as one goes on we shall stop at this point and merely note that we have in a rather painless way obtained the general expressions for the eigenvalues of $\tilde{\mathbf{P}}_N$ which are, of course, also the eigenvalues of the matrix $\mathbf{P}_N$. The series of eigenvalues which one obtains upon substituting integers ranging from 0 to N in equation (10) has the form:

$$\lambda_0 = 1,\ \lambda_1 = 1,\ \lambda_2 = \frac{2(N-1)}{2N-1},\ \lambda_3 = \frac{2^2(N-1)(N-2)}{(2N-1)(2N-2)},$$

$$\lambda_4 = \frac{2^3(N-1)(N-2)(N-3)}{(2N-1)(2N-2)(2N-3)}, \text{ etc.} \tag{10}''$$

We see that $\lambda_2 = \frac{2(N-1)}{2N-1}$ corresponds to the number which we have heretofore designated as λ_L. Substituting λ_2 into equation (7)′ we get the following simple result:

$$\frac{I^{\alpha-1}-I^{\alpha}}{I^{\alpha-1}} \cong \frac{1}{2N-1} \qquad \text{for } \alpha \text{ large} \tag{11}$$

By use of the experimental determination of $\frac{I^{\alpha-1}-I^{\alpha}}{I^{\alpha-1}}$ at equilibrium the value of N obtained from equation (11) turns out to be N = 45. For this value of N, none of the schemes devised for determining the eigenvectors (see for example the comment following equation (10)′) could be used with ease. Also raising the matrix $\mathbf{P}_N$ to various powers could not be accomplished readily by hand. Accordingly, it was decided to carry out the computations on a high speed digital computer. The MIDAC (Michigan Digital Automatic Computer) was chosen because of its availability. The computer was commanded to compute and print out the quantities ${}^{k}p^{\alpha}_{45}(0)$ for k ranging from 1 to 44 and α ranging from 0 to 200. The machine was also programmed to calculate two other cases N = 9 and N = 15. A few cases for lower N were worked out by hand. It was hoped that by a comparison of the results for ${}^{k}p^{\alpha}_{N}(0)$ and ${}^{k}p^{\alpha}_{N}(N)$ for various values of N useful approximate formulas for general N could be obtained. As yet little concrete work along these lines has been done. From the symmetry of $(\mathbf{P}_N)^{\alpha}$ (the first row of $(\mathbf{P}_N)^{\alpha}$ reading to the right is the same as the last rwo of $(\mathbf{P}_N)^{\alpha}$ reading to the left) the quantities ${}^{k}p^{\alpha}_{45}(45)$ for k = 1, 2, ... , 44 can be obtained from a knowledge of the quantities ${}^{k}p^{\alpha}_{45}(0)$, k = 1, 2, ... , 44. From ${}^{k}p^{\alpha}(0)$ and ${}^{k}p^{\alpha}(N)$, ${}^{k}I^{\alpha}$ may be computed. From the machine-results families of curves for the ratios $\frac{{}^{k}p^{\alpha}(0)-{}^{k}p^{\alpha-1}(0)}{I^{\alpha-1}}$ and $\frac{{}^{k}p^{\alpha}(45)-{}^{k}p^{\alpha-1}(45)}{I^{\alpha-1}}$ have been computed to serve as a basis for comparison with experimental results (see figure 1). Since the eigenvector of $\mathbf{P}_N$ corresponding to the leading eigenvalue is even about its middle row the above ratios both approach, as α becomes large, the limit $\frac{1}{2}(1-\lambda_L) = \frac{1}{2(2N-1)}$.

We conclude with a few remarks which might be termed second-sight observations. First the eigenvectors of $\mathbf{P}_N$ corresponding to the non-degenerate eigenvalues are either even or odd. For if one has an eigenvector which is neither even nor odd then one can by reversing its components (putting the N^{th} component in place of its 0^{th}, its N − 1 instead of its first, etc.) produce a new vector which is distinct from the old. From the symmetry of $\mathbf{P}_N$ ($\mathbf{P}_N$ is not what is called a symmetrical matrix; i.e., $\mathbf{P}_N \neq \widetilde{\mathbf{P}}_N$. However, it has many interesting symmetries. The particular symmetry referred to here is the fact that successive rows from the top read to the right are the same as successive rows from the bottom read to the left.) This too would be an eigenvector of $\mathbf{P}_N$ corresponding to the same eigenvalue and hence we would have a contradiction with the assumption that we are dealing with a non-degenerate eigenvalue. The leading eigenvector of $\mathbf{P}_N$ may

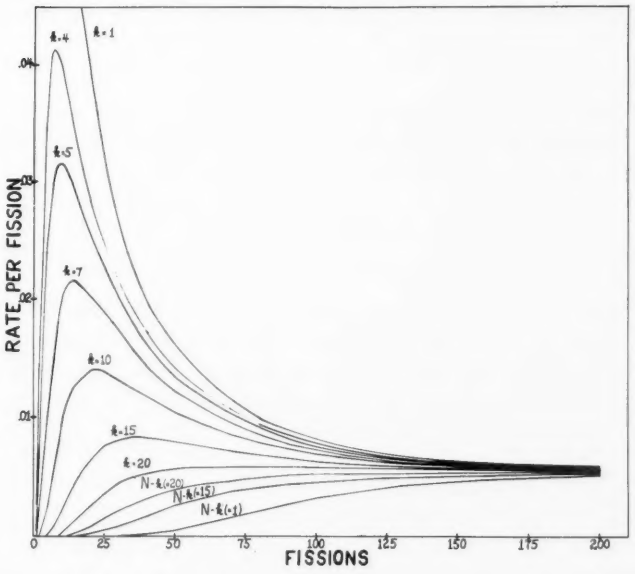

FIGURE 1. Family of theoretical curves of different values for N = 45.

be shown to be even and it is strongly suspected although not proven that the succeeding eigenvectors are alternately odd and even. With such a situation it may be concluded that the ratio $\frac{k_I{}^{\alpha-1} - k_I{}^{\alpha}}{k_I{}^{\alpha-1}}$ would reach its limiting value sooner than either $\frac{k_P{}^{\alpha}(0) - k_P{}^{\alpha-1}(0)}{k_I{}^{\alpha-1}}$ or $\frac{k_P{}^{\alpha}(N) - k_P{}^{\alpha-1}(N)}{k_I{}^{\alpha-1}}$ as the former contains only sums over all the "impure" fractions and therefore would contain only the even terms.

The evenness of the leading eigenvector may be used to advantage in connection with the calculation of the relative fractions of cells corresponding to the various impure types to be expected for large α. This distribution may be seen from equations (4)′, (4)‴ and (6)′ to be, for all values of k given by the N − 1 components of the leading eigenvector, χ_L, of $\mathbf{P}_N$ which have reference to the impure types, i.e., the second through to the next to the last components of χ_L. In what follows we shall find it convenient to use the N − 1 by N − 1 matrix $\mathbf{P}'_N$ which we define as the matrix $\mathbf{P}_N$ minus its first and last rows and columns and the N − 1 component vectors χ_1' which are obtained from the eigenvectors χ_1 of $\mathbf{P}_N$ by slicing off their first and last components. Because of the nature of $\mathbf{P}_N$, (to be specific, it has all zeros in rows 1 through N in the first column and all zeros in rows 0 through N − 1 in the last column) if χ_1 is an eigenvector

of $\mathbf{P}_N$ corresponding to λ_1, χ_1' is an eigenvector of $\mathbf{P}_N'$ corresponding to λ_L. Now successive applications of $\frac{1}{\lambda_L}\mathbf{P}_N'$ to any N − 1 component vector q will produce a vector which is proportional to χ_L'. This may be seen directly by expanding q in terms of the χ_1''s. We have: $q = \Sigma \alpha_1 \chi_1'$ where the α_1's are constants.

$$\left(\frac{1}{\lambda_L}\mathbf{P}_N'\right)^m q = \Sigma \alpha_1 \frac{\lambda_1}{\lambda_L^m} \chi_1' \cong \alpha_L \chi_L' \qquad \text{for m large enough}$$

The better the initial choice of q the more rapidly will this process converge. Since χ_L is known to be even an initial choice of q as an even vector will hasten the convergence of the procedure. Even for as large a value of N as 45, it is estimated that the computation of χ_L' should require only several days work on a good hand computer.

ACKNOWLEDGEMENTS

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THE RESISTANCE OF STRAINS OF D. MELANOGASTER TO DDT AND DIELDRIN.*

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INTRODUCTION

DDT and several other insecticide resistances of strains of *D. melanogaster* were investigated by Bartlett (1952) who found that the effect of selection by DDT was consistently higher in original field-collected strains than in other relatively isogenic laboratory strains. From this result, the original field-collected strains were thought to be heterogeneous in genetic factors for resistance. The presence of these may or may not have arisen through previous contact with DDT.

The character of insecticide resistance has been generally presumed by geneticists to be of a polygenic and pre-adaptational nature, therefore to find the variations of this complex genetic system in populations would be very interesting. The history of strains used in this experiment except for Hikone, a Japanese strain, concerning previous selection for any insecticide was not known, but they were so extremely heterogeneous in resistance to DDT and to dieldrin (DL) that genetic variation within the original populations can be assumed.

MATERIALS AND METHODS

Eighteen strains from ten localities were investigated for their levels of resistance to DDT and eleven strains from five localities were used for detecting levels of resistance to DL (see table 1).

Strains started by different females captured in a single collection at each geographic locality are identified by numerical subscripts. These strains except for Hikone have been cultured by mass-mating at Cold Spring Harbor; Hikone has been cultured in Japan. The H strain was started from several females captured at the small town of Hikone, where DDT and several other insecticides had been used for killing mosquito larvae for five years beginning in 1946, and its resistance not only to DDT, but also to BHC and parathion has been reported by Tsukamoto and others (1954, 1956 and 1957).

*This work was performed while the author was a Rockefeller Foundation Fellow.

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TABLE 1

ANNOTATED LIST OF THE STRAINS OF *D. MELANOGASTER* USED IN DDT AND DL RESISTANCE STUDIES

Symbol	Strain name and place of origin	Date of capture
†RC_2 †RC_9	Riverside, California, U.S.A.	March, 1954 from a small citrus grove
†BS_{10} †BS_{11}	Barcelona, Spain	June, 1954
*CA_2 †CA_9 *†CA_7 *CA_{12}	Capetown, South Africa	May, 1954 from Stellenbosch near Capetown
†BER_1 *†BER_3	Bermuda	August, 1954
†QI_{10} †QI_3	'Qiryath, Israel	April, 1954 from a fruit-growing district 10 kms from Jerusalem
*†BV_{11} †BV_1 *BV_4 *BV_8	Blacksburg, Virginia, U.S.A.	March, 1954 from a grocery store in Blacksburg
†NO_1 †NO_2	New Orleans, Louisiana, U.S.A.	March, 1954
*†SC_7 †SC_5 *SC_{11} *SC_{12}	Santiago, Chile	1954 from a local vineyard
†SY	Syosset, New York, U.S.A.	July, 1952 from a grocery store
*†H	Hikone, Japan	September, 1952

†DDT only.
*DL only.
*†Both.

Several pairs of flies of each strain were placed in a bottle containing corn meal, molasses, agar and yeast medium, were allowed to lay eggs for three days, and then were transferred into a second bottle. Female offspring were etherized and divided into several groups of forty flies each. These groups of flies were aged for 2–3 days in vials (diameter 2.5 cm., length 10 cm.) containing the same medium before testing. The forty female flies, 4–5 days old, were transferred without etherization to vials containing a sheet of DDT, DL or control test paper (5 × 6 cm.). The test papers were prepared by the WHO in Geneva; their specifications are as follows:

DDT test paper	Concentration 4.0%	357 mg/100 cm^2
	Impregnation date:	29 Mrz. 1957
	Batch No. 34	Control No. 01475

DL test paper	Concentration 0.8%	357 mg/100 cm²
	Impregnation date:	18 Mrz. 1957
	Batch No. 28	Control No. 00729
	Concentration 0.4%	357 mg/100 cm²
	Impregnation date:	20 Mrz. 1957
	Batch No. 27	Control No. 00539
Control paper	Concentration 0% insecticide	357 mg/100 cm²
	Impregnation date:	2 Apr. 1957
	Batch No. 35	Control No. 01756

These test vials were kept in a dessicator at a humidity of 75% and a temperature of 25°C. The exposure time to DDT test paper was either 5, 10 or 15 hours, to DL test paper, 0.5, 1.0, 2.0 and 3.5 hours, and to control paper, 15 hours. After exposure, flies were transferred into a fresh vial containing a wet filter paper (5 × 6 cm., impregnated with 0.4 ml. distilled water) and the numbers of dead flies were scored at the time of transfer and 10, 20, 25, 35, 45, 50 and 60 hours later. These vials were kept in a room in which the humidity was about 50% and temperature 25°C. The accumulated mortalities at these times were recorded. These tests were repeated three times using female flies obtained from the sub-cultures of each strain.

EXPERIMENTAL RESULTS

1. Control experiment.

The cumulative mortality curves for each strain after exposure to control paper for 15 hours are represented in figure 1. These curves were obtained by averaging the mortalities of the three replicates. Flies in most strains did not die until 25 hours after exposure, but flies of the BS_{11} strain started dying after only 10 hours. In striking contrast, most flies of the H strain were not dead even after 60 hours. Under these conditions, flies of most strains began to die between 25 and 35 hours as the result of desiccation and starvation.

The numbers of dead flies 50 hours after exposure were analyzed statistically as to locality and strain; the SY and H strains were omitted from the calculations. The results are shown in table 2.

These results indicate that the difference between strains was significant but that the difference between localities was not.

TABLE 2

ANALYSIS OF VARIANCE OF NUMBERS OF DEAD FLIES 50 HOURS AFTER EXPOSURE TO CONTROL PAPER FOR 15 HOURS

	S.S.	d.f.	M.S.	F
Locality	972	7	138.9	1.97
Strain	1661	8	207.6	2.94*
E	2191	31	70.7	

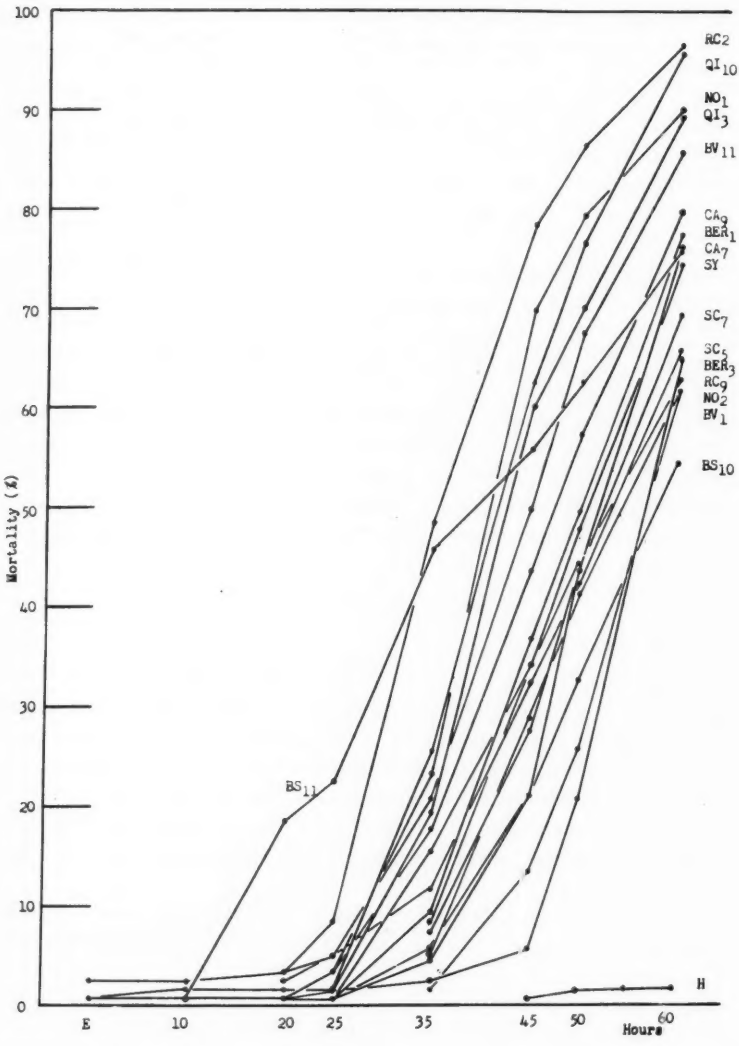

FIGURE 1. Cumulative mortality curves of 18 strains after exposure to control paper for 15 hours.

2. DDT-resistance of strains.

The cumulative mortality curves of each strain after exposure to 4 per cent DDT test paper are shown in figure 2. Each curve was calculated on the basis of the mean mortalities of the three replicate experiments. A "delirium tremens" condition (DDT's) was found among flies of most non-

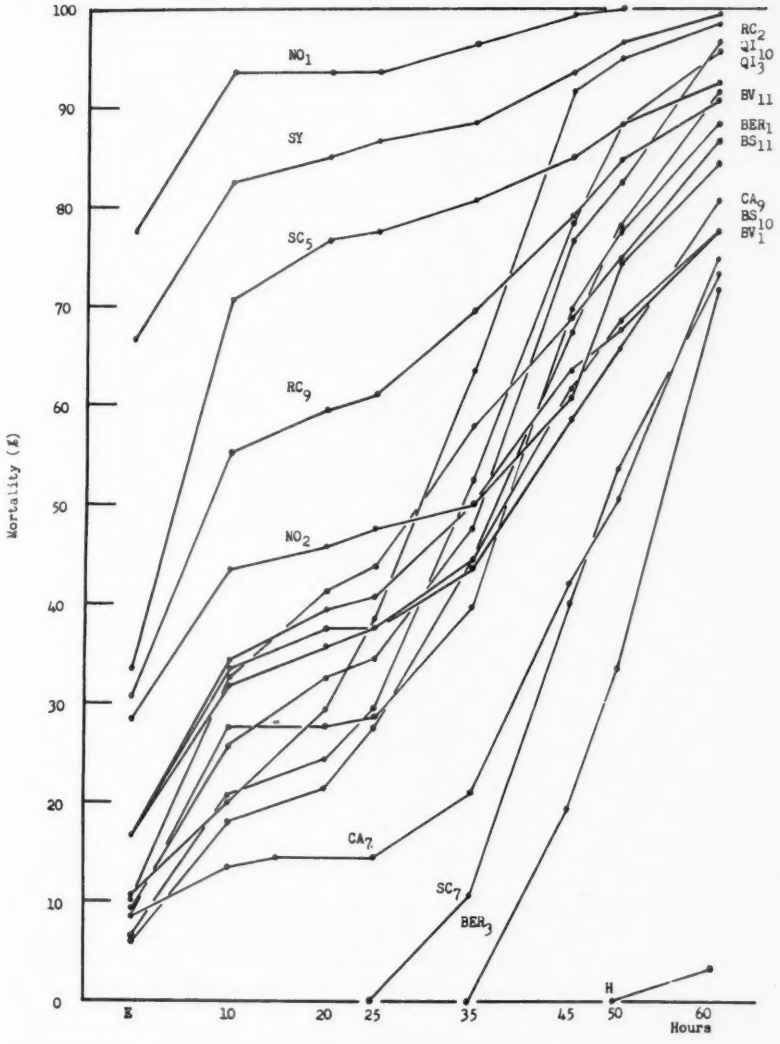

FIGURE 2. Cumulative mortality curves of 18 strains after exposure to 4% DDT test paper for 15 hours.

resistant strains after exposure and most of these died within ten hours. The curves remain almost horizontal for the next 15 hours and then rise once more. As in the control experiments, the increase in mortality after 25 hours is assumed to be caused by desiccation and not by poisoning. Accordingly, the levels of DDT-resistance of strains were decided on the basis of percentage mortality 25 hours after exposure to DDT test paper.

TABLE 3

CUMULATIVE MORTALITIES OF 18 STRAINS OBSERVED 25 HOURS AFTER EXPOSURE TO 4% DDT TEST PAPER

Strain	Exposure time (Hours) 5	10	15
RC_2	0	26.7	38.3
RC_9	5.9	11.7	61.0
BS_{10}	2.5	18.3	40.7
BS_{11}	2.5	29.2	43.6
CA_9	6.7	46.6	37.7
CA_7	1.7	10.8	14.3
BER_1	0	10.2	28.7
BER_3	0	0	0
QI_{10}	0	22.0	34.2
QI_3	0	6.7	29.2
BV_{11}	0	12.6	27.5
BV_1	3.8	12.5	37.5
NO_1	7.5	66.4	93.6
NO_2	0	45.0	47.5
SC_7	0.9	0	0
SC_5	7.5	30.0	77.5
SY	10.8	30.5	86.7
H	0	0	0
Overall avg. (%)	2.8	21.1	38.7
Total number of flies tested	2064	2103	2137

The accumulated mortalities of 18 strains 25 hours after exposure to 4% DDT test paper for 5, 10 and 15 hours are shown in table 3.

The three points representing total average mortality at different time doses are linear on normal logarithmic section paper. Resistance to DDT thus appears to be normally distributed in populations.

The numbers of dead flies of 16 strains of 8 localities not including the SY and H strains 25 hours after exposure to 4% DDT test paper for 15 hours were analyzed statistically as to locality and strain. The results are given in table 4.

Highly significant differences in DDT-resistance between localities and between strains were found; the latter was larger than the former but not significantly so.

TABLE 4

ANALYSIS OF VARIANCE OF NUMBERS OF DEAD FLIES 24 HOURS AFTER EXPOSURE TO 4% DDT TEST PAPER FOR 15 HOURS

	S.S.	d.f.	M.S.	F
Locality	1740	7	248.6	5.45**
Strain	2288	8	286.0	6.27**
E	1460	32	45.6	

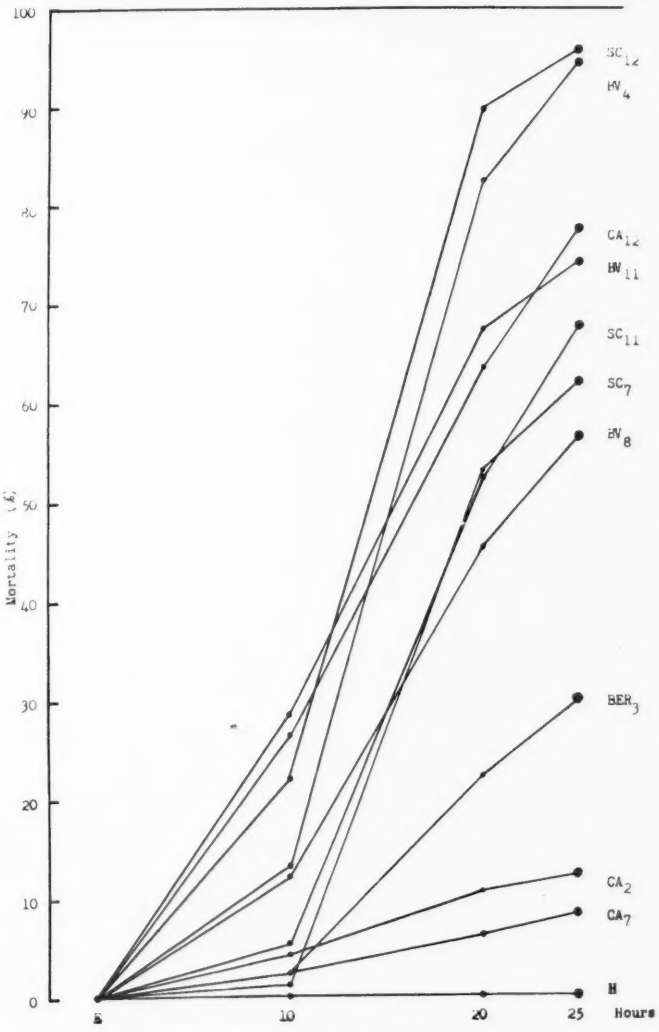

FIGURE 3. Cumulative mortality curves of 11 strains after exposure to 0.8% Dieldrin test paper for 30 minutes.

3. DL-resistance of strains.

The cumulative mortality curves of 11 strains representing 5 localities after exposure of 0.8 per cent DL test paper for thirty minutes are shown in figure 3.

Each curve is based on the mean mortalities of three replicate experiments. In this case, the exposure time was short and therefore "delirium

TABLE 5

CUMULATIVE MORTALITIES OF 11 STRAINS 25 HOURS AFTER EXPOSURE TO 0.8, 0.4% DL TEST PAPER

DL dose	0.8%				0.4%			
	Exposure time (Hours)							
Strain	0.5	1.0	2.0	3.5	0.5	1.0	2.0	3.5
BV_4	94.3	...	...	...	12.7	98.3	100.0	100.0
BV_8	56.9	...	...	...	12.5	56.7	95.8	100.0
BV_{11}	74.2	...	...	...	11.8	81.4	94.1	99.2
CA_2	12.2	...	...	...	0	3.4	26.7	96.2
CA_7	8.2	41.2	92.4	100.0	0.8	0	10.0	71.6
CA_{12}	77.5	...	...	...	13.3	58.3	97.5	100.0
SC_7	62.0	85.7	100.0	100.0	1.7	66.7	97.5	100.0
SC_{11}	67.9	...	...	...	27.5	82.5	99.2	100.0
SC_{12}	95.6	...	...	...	55.8	100.0	99.2	100.0
BER_3	30.0	100.0	100.0	100.0	0	0.8	79.8	100.0
H	0	1.7	91.8	98.3	0	0.8	0.8	17.6
Overall avg. (%)	52.6				12.4	49.9	72.8	89.5
Total no. of flies tested	1748	478	476	475	1308	1312	1311	1315

tremens" appeared about ten hours after exposure in most non-resistant strains. The mortality continues to increase after that time.

The percentages of accumulated mortalities for these strains 25 hours after exposure to 0.4 or 0.8 per cent DL test paper for 0.5, 1.0, 2.0 and 3.5 hours are shown in table 5.

The four points representing total average mortality at different time doses after exposure to 0.4 per cent DL test paper are nearly linear on normal logarithmic section paper. Consequently, resistance to DL appears to be normally distributed in populations just as is DDT-resistance.

The numbers of dead flies of 9 strains representing 3 localities, not including the BER_3 and H strains, 25 hours after exposure to 0.8 per cent DL test paper for one half hour were analyzed statistically as to locality and strain. The results are given in table 6.

TABLE 6

ANALYSIS OF VARIANCE OF NUMBERS OF DEAD FLIES 25 HOURS AFTER EXPOSURE TO 0.8% DL TEST PAPER FOR HALF AN HOUR

	S.S.	d.f.	M.S.	F
Locality	2267	2	1133.5	15.44**
Strain	2781	6	463.5	6.31**
E	1981	27	73.4	

Highly significant differences in DL-resistance between localities and between strains were found but, in this case, the variation between localities is significantly larger than that between strains.

4. Cross-tolerance to DDT and DL.

From the results mentioned above, the strains used in this experiment were classified into three groups and arranged by their levels of resistance to DDT and DL as follows:

	DDT	DL
Resistant	H	H
	BER_3	CA_7
	SC_7	CA_3
	CA_7	BER_3
Slightly resistant	BV_{11}	BV_8
	QI_3	SC_7
	BER_1	SC_{11}
	QI_{10}	BV_{11}
	BS_{11}	CA_{12}
	RC_2	
	CA_9	
	BV_1	
	BS_{10}	
Non-resistant	NO_2	BV_4
	RC_9	SC_{12}
	SC_5	
	SY	
	NO_1	

The H strain has the greatest resistance to both insecticides; other relatively resistant strains do not exhibit this cross resistance so clearly.

The CA_7 and SC_7 strains, resistant to DL and DDT respectively, were crossed and the levels of resistance to DL of F_1 and F_2 hybrids were determined. Their levels were similar to that of the resistant parental strain CA_7 and no difference between reciprocal crosses was found. Twenty pairs were taken at random from the F_2 hybrid flies and the levels of resistance to DDT and DL of their offspring were tested. The F_3 individuals from some pairs showed resistance to both insecticides; others showed resistance to one; and still others were sensitive to both. These results indicate that the variation in F_2 hybrids was great and that resistance to DDT and to DL are different polygenic characters, which have some common factors in their genetic systems. Additional information regarding the complexity of these genetic systems is given by the H and BER_3 strains which are resistant to both insecticides and to desiccation; this correlation was not found in the other strains studied.

DISCUSSION

The nature of the genetic systems responsible for resistance to DDT and DL in natural populations cannot be completely determined by the

experimental data described above. When we consider the strains tested, we should recall not only their diverse geographic origins but also their subsequent maintenance by mass-culture for several years in the laboratory. Nevertheless, we may safely conclude from the data that natural populations of Drosophila are extremely heterogeneous for resistance factors.

Bartlett (1952) selected for resistance to DDT within field-collected strains and relatively isogenic laboratory strains such as Canton-S; he found that the former showed a marked response to selection but that the latter remained relatively susceptible to this insecticide. Crow (1954) obtained by selection a strain highly resistant to DDT from a mixed population composed of many laboratory strains. King (1958) selected a laboratory Oregon-R strain and a field-collected Syosset strain by a DDT-aerosol method and found in general that the latter responded faster than the former. In one of several Syosset strains, resistance by the fiftieth generation had reached a level measured in terms of LD_{50} twenty times that of the control. This level was maintained for forty generations. These facts indicate that a successful response to selection depends on genetic heterogeneity. The H strain was found to be resistant to DDT when originally collected. This is the only resistant strain of Drosophila which has arisen in the field. In the laboratory this strain has been subjected to further selection for DDT resistance but with no pronounced response. Tsukamoto and Ogaki (1954) determined the locus of a second-chromosome gene responsible for resistance, but their arguments concerning dominance of this gene are not conclusive. Oshima has recently checked the genetic factors on the second chromosome of the H strain. CyL/Pm female flies were crossed with Hikone male flies and several F_1 hybrid CyL male flies were backcrossed individually with several CyL/Pm female flies. Several pairs of CyL flies obtained from each F_2 culture were mated, wild type F_3 flies homozygous for given second chromosomes of the Hikone strain and CyL flies heterozygous for the same chromosomes were obtained. Upon testing their resistance to DDT, the factors on different second chromosomes were found to be remarkably different. These experimental data demonstrate that the genes for resistance located on the second chromosome may be heterogeneous even within the Hikone population.

Deiner and Crow (1951) reported that a DDT-resistant strain of *D. melanogaster* showed cross-resistance to certain chlorinated hydrocarbons such as BHC, toxaphene, methoxychlor and aldrin. Oppenoorth and Dresden (1953) described an instance of cross-resistance between DDT and Thanite in a BHC-resistant strain. Tsukamoto and Ogaki (1954) observed a cross-resistance between DDT, BHC, parathion and nicotine sulfate in a resistant Hikone strain, but Tsukamoto and Hiroyoshi (1956) proved that the resistance to nicotine sulfate was primarily the result of a dominant gene on the right arm of the third chromosome. Tsukamoto (1957) suggested that DDT, BHC, and Dipterex resistance in Drosophila might be controlled by a common factor on the second chromosome. Metcalf (1955) has reported that

house flies which developed resistance solely to DDT had only a slight degree of resistance to lindane, dieldrin and related chlorinated hydrocarbons. The slightly higher resistance to the latter compounds has been attributed to an increased vigor of the strain.

In our experiment we have assumed the existence of specific as well as common factors for resistance to DDT and DL. At any rate, the resistance to insecticides is a polygenic character not unrelated to viability and, in a natural population, it is part of an extremely heterogeneous genetic system.

The statistical analysis of DDT-resistance revealed that there was a larger within-locality than between-locality variance; on the contrary, the latter was found to be significantly larger in the case of DL-resistance. Whether this is a reflection of the worldwide use of DDT and a more restricted use of dieldrin at the time our strains were collected is not known.

SUMMARY

1. The level of DDT-resistance of eighteen strains of *D. melanogaster* collected from ten localities in Europe, Africa, Asia and North and South America was tested by exposure to 4 per cent DDT test paper prepared by the WHO in Geneva. The level of dieldrin resistance of eleven strains of five localities was tested by exposure to 0.8 and 0.4 per cent dieldrin test paper.

2. The total average mortalities of these strains after exposure for varying times were almost linear when plotted on normal logarithmic section paper. The expression of resistance to DDT and DL is, therefore, normally distributed in populations.

3. From the results of analyses of variance using numbers of dead flies 25 hours after exposure to DDT, DL and control test papers, significant differences in resistances to both insecticides were observed between localities and between strains. Significant differences between strains but not between localities were found in resistance to desiccation.

4. Cross-resistance between DDT and DL was observed; it was concluded that these resistances are the result of different genetic systems of which some factors are common to both.

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BIRD PREDATION AND FOODPLANT SPECIFICITY IN CLOSELY RELATED PROCRYPTIC INSECTS

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The study of foodplant preferences in phytophagous insects has for some time been a subject of active physiological research directed towards discovering the ways by which insects select specific plants in their natural environment. Dethier (1954), Fraenkel (1953), and Painter (1953) have each stressed that the nutritional suitability of the plant species plays a subsidiary role in determining foodplant choice and that nutritionally unimportant stimuli (attractant and repellant compounds in the leaves) are predominantly responsible for regulating feeding preferences. In partial contrast to this view is that of Kennedy (1953) who maintained that even though these stimuli do play a role in directing foodplant choice, insects also choose by means of sensory mechanisms directly related to determining the nutritive value of plants.

These two views both relate to how foodplant preferences operate but neither bears on the problem of why some insects have come to be restricted in their diets while others have not. In discussing this problem, Dethier accepted the view of several earlier authors that polyphagy represents the primitive state out of which restricted feeding has evolved and he presented various possible lines leading to oligophagy or monophagy. Although Dethier and Kennedy each alluded to ecological competition with other species of insects as a selective force in producing foodplant specialization, neither discussed this aspect to any extent. In fact both Dethier and Painter emphasized that the evolution of feeding preferences in insects has been governed by the evolution of resistant mechanisms in the plants, and the converse. The purpose of this paper is to show how procryptic insect individuals feeding together on plants can interact, and to discuss how this interaction can lead to a specialized or a generalized foodplant diet.

One of the reasons why ecological competition between phytophagous insects has received so little attention as a factor implementing preferences is undoubtedly related to the well known fact that under natural conditions most leaf-eating insects do not regularly defoliate the plants on which they feed. Hence while food shortage sets a potential upper limit to the numbers of phytophagous insects, this limit is reached only occasionally. However, as Nicholson (1927) pointed out, the numbers of any phytophagous insect species do bear a relationship to the quantity of food available, because

an increase in foodplant in any given area is usually accompanied by an increase in the number of insects even though in each case there is an ample surplus of food. He further suggested that the intensity of attack delivered by the natural enemies of the insects increases as they are more easily found and that the ease with which they are found is determined by their density on the foodplants. In this way, their mortality would be density dependent, that is, a constant number of enemies would discover and kill an increasingly greater percentage of the insects as the latter increased in density.

Recent studies both in the laboratory and in the field have shown how this relationship may be brought about by bird predators even though Lack (1954) believed that birds do not exert an effective control on insect numbers. In a series of experiments carried out by de Ruiter (1952), twig-like geometrid caterpillars (*Ennomos alniaria* L., *Biston hirtaria* Cl., and *B. strataria* Hufn.) and twigs which they specifically resemble were scattered over the floor of an aviary. Individual Jays (*Garrulus glandarius* Hartert) were then admitted and characteristically began to hop about looking for food but in each case ignored both the twigs and the motionless caterpillars. However, after four out of eight birds finally found and ate a larva either because it moved or because the experimenter lured the bird next to it with bread crumbs, both twigs and larvae were pecked at which resulted in nearly all of the larvae being eaten. Moreover, in discussing these experiments, Tinbergen (1957) said that when caterpillars outnumbered twigs, the birds went on hunting for caterpillars, but if they picked up more twigs than caterpillars, they gave up searching. From this it can be seen that the time interval between successes can not be too long if the bird is to continue searching, and in nature this interval would bear a direct relationship to how well the prey was spread out (Tinbergen, 1952). In experimental studies on cryptic coloration in the melanic and non-melanic forms of the moth *Biston betularia* L., Kettlewell (1955) has found that individuals which harmonize with their background enjoy a selective advantage in that they escape from bird predation more often than contrasting ones do. However, in aviary experiments a pair of Great Tits (*Parus major* Pražak) which at first took a low proportion of the harmonizing individuals later began finding them all. In this same paper, Kettlewell also reported direct observations of a Hedge Sparrow (*Prunella modularis* (Hartert)) and a Robin (*Erithacus rubecula* Hartert) predating moths which he had released on tree trunks in the field. From these observations it appeared that when a contrasting moth had been found, others in the immediate vicinity were at a disadvantage even if they harmonized with their background. This phenomenon of persistent and successful searching after finding the first moth was again directly observed in Redstarts (*Phoenicurus phoenicurus* (L.)), and in a quantitative experiment with admittedly few numbers, it was confirmed that a higher proportion of harmonizing individuals was predated if contrasting

ones were present with them on the tree trunks than if the harmonizing ones were alone (Kettlewell, 1956). In addition to these observations and experiments, Lack concluded that most bird species are known to concentrate on a small number of food items even though they take others occasionally, and he also gave evidence that some birds, while concentrating on a particular species of insect prey, will only select individuals above a certain size.

From these findings it is perhaps not unreasonable to generalize that the behavior of birds which eat phytophagous insects is such that the probability of discovering a food item of a particular shape, color-pattern, and size is increased if the one previously found is of similar appearance and is palatable. In fact, according to Tinbergen, the phenomenon of developing a "searching image" of a specific prey has been observed in many animals. This kind of behavior in birds could easily lead to density-dependent predation in nature. Other factors which could increase the likelihood that the predation would be density-dependent are that some birds are known to restrict their food-hunting activities to particular species of trees, and to particular parts of these as well (Lack, MacArthur, 1958). Moreover, many insects restrict their feeding to limited regions of plants. And finally, differences between individuals of a single plant species can lead to a concentration of insects on some, and their absence or scarcity on others (Varley, 1957; Brower, ms. in preparation).

These considerations are important from the point of view of competition between closely related species of procryptic insects feeding together on the same plants because of the fact that when newly evolved species meet for the first time after their origin in geographic isolation they will often be similar in appearance. As Mostler (1935) and Jane Brower (in press) have shown, various passerine birds trained to avoid distasteful insects will also refuse on sight alone insects which bear a general resemblance to them. On the basis of these experiments it can be predicted that a bird in nature would *often* fail to distinguish one of the closely related species of insects from the others, that is, it would treat them all as one "visual species." When the new species began to penetrate the ranges of each other, there would be a tendency for each to increase its numbers up to the level where it occurred alone. Because the birds would usually fail to discriminate one from the others, a searching image developed on any one would include them all and the effective density of each individual species would be multiplied by the number of species feeding together on the same foodplants. This combined density might then be high enough to trigger heavy predation as in de Ruiter's stick caterpillars. In this way the birds could exert an important control on the numbers of each of the species present and thereby prevent them from becoming widely sympatric.

But even if the combined density of the closely related species were too low for the predation to be effective in controlling their population sizes,

the presence of individuals of any one of the species would still increase the probability of death of individuals in all the species. However, it is almost certain that this adverse interspecific effect would be differential because the genetic differences among them would result in each being cryptic to a slightly different extent on any one foodplant species. Because of this, the selection pressure exerted by the birds concentrating on the common prey image would favor those individuals of each species which were on mutually exclusive plants, and in this way the common foodplant diet originally shared by all would come to be divided among them. Oligophagy could thus arise from polyphagy. Moreover, mutually exclusive oligophagy could similarly evolve in insects which were not closely related provided they were of similar appearance. The reason that foodplant specialization is so prevalent is probably because the selective advantage of being on separate plants is greater than that conferred by the initial stages of a divergence in appearance which would ultimately be different enough to be overlooked by the birds.

It should also be noted that if only one of the closely related species were present in an area, polyphagy would be at a selective advantage because it would scatter the same number of individuals over several plant species rather than concentrating them on one or a few. Alternatively, or in conjunction with this, there is the possibility that a polymorphism of color-pattern characters could develop which would break up the population into several different "visual species," each of which would be a separate prey image to be learned and searched for by the birds. Such a polymorphism could be maintained because the rarest of the forms would always be at the greatest advantage (Wright and Dobzhansky, 1946; Sheppard, 1958; Williamson, in press).

From the considerations discussed above, it is concluded that food preferences in phytophagous insects which are procryptic and palatable to birds are in part an expression of the sympatry or allopatry of closely related species. Moreover, it would seem advisable to reexamine the generalization that polyphagy is primitive and oligophagy specialized, for as the process of geographic isolation, speciation, and subsequent range expansion leading to sympatry repeats itself, the insects could alternate between these two states.

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LETTERS TO THE EDITORS

Correspondents alone are responsible for statements and opinions expressed. Letters are dated when received in the editorial office.

RELATIVE TIME OF ECLOSION OF DROSOPHILA FEMALES HETEROZYGOUS FOR SEX-LINKED RECESSIVE LETHALS*

In Drosophila, genes that are lethal in homozygotes or hemizygotes commonly produce an impairment of viability in heterozygotes[1], although some exceptions which apparently give increased viability have been reported[2,3,4]. It could be expected that some lethals would have beneficial heterotic effects leading to increased viability; this is especially true in view of the work of Wallace[5], who has shown that even newly originated mutant genes had average heterotic effects before selection had a chance to operate.

Lethal genes in heterozygotes no doubt exert their influence on a variety of physiological processes, any one of which would affect the fitness of the individual. Stern et al[4] have shown that some lethals change the rate of development of heterozygous females, so that relative viability may be a reflection of developmental rate, as well as survival. If most lethals are deleterious in heterozygotes, and if slowing down the developmental rate is one mode of action, it could be predicted that females heterozygous for sex-linked recessive lethals would tend to eclose later than their normal sisters. However, if most of the lethals are neutral in their action, no effect on eclosion would be noted; and if heterotic effects predominate, earlier eclosion of heterozygotes would be anticipated. To test this, during the course of a study[6] involving recovery of large numbers of radiation-induced sex-linked recessive lethals, the emerging female heterozygotes were collected in such a way that the relative rates of emergence of females heterozygous for lethals, *versus* their non-lethal sibs, could be compared. The results are presented here.

Male *Drosophila melanogaster* (Oregon-R strain) were irradiated with 3200 rads of 1.6 mev electrons from a Van de Graaff accelerator, and mated to Muller-5 females (complete details of irradiation and matings will be found in reference 6). The F_1 females tested were the products of mass cultures. Temperature during the experiment was nearly constant at 73°F; and emergence of females usually began on the 11th day after removal of the P_1 females. Collections were made every morning and evening (at 12-hour intervals) until each culture was exhausted. Collections from each culture vial were serially numbered, beginning with the first successful

*The opinions or assertions contained herein are those of the writer and are not to be construed as official or reflecting the views of the Navy Department or the Naval Establishment at large.

collection. The culture usually was exhausted after four collections had been made, but occasional females were collected until as late as the eighth collection period. With few exceptions, these latest emerging females died without progeny or were sterile. Each F_1 female was tested individually for the occurrence of a sex-linked recessive lethal in the irradiated X-chromosome, by the usual Muller-5 technique. In doubtful cases, F_2 females were retested.

The ratio of lethal chromosomes to the total number of chromosomes tested is given in table 1. Each value in the table represents the sum of four replications. The value for collection period 4 includes the small number collected in subsequent periods (through period 8). The differences in lethal frequency between the four collection periods are highly significant ($P < 0.01$).

TABLE 1

PROPORTION OF LETHAL X-CHROMOSOMES COLLECTED IN SUCCESSIVE PERIODS

Chromosomes tested	Collection Period				Sum
	1	2	3	4+*	
Lethal	105	255	165	84	609
Total	1669	3080	1729	831	7309
% Lethal	6.29	8.28	9.54	10.11	8.33

*Includes all females collected subsequently.

The probability that an emerging female possesses a lethal X-chromosome obviously increases with each successive collection period. This proportionality can be estimated by assuming that the response is linear over the interval of collecting. Subjecting the results in Table 1 to a weighted linear regression analysis, the equation obtained for the regression line is $Y = 5.342 + 1.338\ X$, where Y is the expected percentage of lethals and X is the sequential collection period. This is presented graphically in figure 1. Having calculated the equation for regression, the assumption of linearity can be tested by a method of partitioning χ^2 given by Cochran[7]. This analysis indicates that the data are adequately described by a linear relationship ($P < 0.01$).

These results indicate that the majority of 609 X-chromosomes containing newly-induced recessive lethals prolonged development, as measured by eclosion, and suggest that at least part of the viability impairment of heterozygous lethals noted by other authors[1,4] is a reflection of a decreased developmental rate. Most lethals seem to be measurably deleterious, rather than neutral or beneficial, when in heterozygotes. In this respect, they can be compared to other types of mutations such as Minutes, which, although they have a dominant phenotypic effect, also result in a marked prolongation of development in heterozygotes, and lethality in homozygotes.

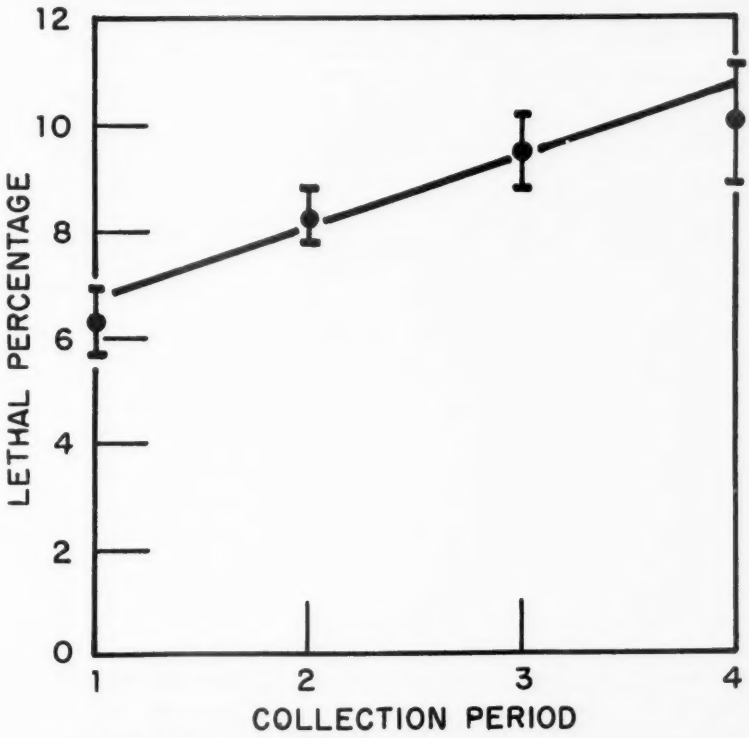

FIGURE 1. Weighted linear regression of recovery of females heterozygous for chromosomes containing newly-induced sex-linked recessive lethal mutations, plotted against the period of collection measured at sequential 12-hour intervals. Abscissa = collection period; ordinate = lethal percentage. The standard deviation is indicated for each experimental point.

It is quite likely that many of the chromosomes tested had, in addition to at least one lethal mutation, one or more non-related mutations induced by the treatment. These may have contributed to the reduction in developmental rate; or, if they exerted beneficial heterotic effects, they were not powerful enough to overcome the deleterious effects of the lethals.

It seems clear that all studies involving collection of flies heterozygous for lethals should include a note on the duration of the collections, since those continuing to culture exhaustion would recover a greater proportion of lethal chromosomes, giving consequently higher values.

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